Division of Pharmaceutical Biosciences

Faculty of Pharmacy

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Profiling Antichlamydial Compounds in Monocyte and Macrophage Cell Models

Maarit Kortesoja

DOCTORAL DISSERTATION

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Abstract

Chlamydia pneumoniae is an obligate intracellular human pathogen whose primary site of infection is the respiratory tract. In addition to respiratory tract infections, such as sinusitis and pharyngitis, *C. pneumoniae* has been related to several chronic inflammatory diseases of which atherosclerosis is the most widely studied. In order to contribute to the pathogenesis e.g. in atheromatous arteries, *C. pneumoniae* must disseminate from lungs to other tissues. This translocation occurs via peripheral blood mononuclear cells (PBMCs), mainly monocytes and macrophages. The presence of *C. pneumoniae* has been reported to induce inflammatory molecule production and alter redox balance in these cells, while also promoting their foam cell formation, migration and adherence. The ability of *C. pneumoniae* to persist inside the PBMCs, and thus become refractory to conventional antibiotics complicates the management of the associated underlying infection in chronic inflammatory diseases. Most *C. pneumoniae* susceptibility studies are currently based on the use of permissive cell lines, such as epithelial and endothelial cell lines in which the bacteria are actively replicating, which leaves the persistent infections unnoticed. Therefore, in addition to new active compounds against *C. pneumoniae*, new methods to study their effects against the persistent chlamydial infection are urgently needed.

In this study, dibenzocyclooctadiene lignans, new antichlamydial compounds originating from berries of medicinal plant, *Schisandra chinensis*, were profiled regarding different aspects of *C. pneumoniae*-PBMC interactions. Human THP-1, a monocyte and macrophage cell line, and a murine macrophage cell line RAW264.7 were used in the studies to examine the effects of the schisandrin lignans on *C. pneumoniae* infection and their influence on host cell responses.

Oxidative stress is the basic pathological mechanism underlying a spectrum of chronic inflammatory diseases, and it largely contributes to the consequences of *C. pneumoniae* infection as well. In this work, *C. pneumoniae* was found to induce proinflammatory cytokine, interleukin (IL)-12 secretion in the human monocytic cell line. It also increased

the intracellular reactive oxygen species (ROS) and nitric oxide (NO) levels in macrophages, and it had an impact on the concentration of glutathione (GSH), the major small-molecule antioxidant, in macrophages. Gene expression analysis of murine macrophages revealed that *C. pneumoniae* suppressed the peroxisome proliferator-activated receptor γ (*PPARγ)* transcription in the cells, which influences lipid metabolism and inflammatory responses in these cells.

Schisandrin lignans had an impact on *C. pneumoniae*-induced alterations in these cell models. Schisandrin B and schisandrin C reduced the elevated interleukin (IL) -12 cytokine levels, as well as the LPS induced IL-6 and IL-12 levels. Schisandrin lignans also affected cellular oxidative balance by elevating the basal ROS levels while simultaneously reducing the ROS or NO levels induced by infection or LPS. The redox balance alteration was also shown within the GSH levels, which were reduced by the lignans in THP-1 monocytes and macrophages but elevated in RAW264.7 cells. Schisandrin B additionally upregulated the transcription of genes involved in GSH synthesis, *GCL* and *GGT-1*. Schisandrin B and schisandrin C also reduced the *C. pneumoniae*-induced macrophage foam cell formation and altered the related expression of *PPARγ* and *ABCA1* genes.

In this work, a new platform for studying the *C. pneumoniae* transfer between lung epithelial cells and phagocytes, is also introduced. The platform can be used in lead compound profiling studies against *C. pneumoniae* infection. Mitogen-activated protein kinase (MAPK) inhibitors were found to inhibit the transfer of the infection, serving as reference compounds in future profiling studies.

This work provides new information about the *C. pneumoniae* infection in monocyte-macrophage cell models, while also offering valuable insight on the antichlamydial lead compounds, dibenzocyclooctadiene lignans. These lignans were shown to suppress the *C. pneumoniae* induced pathological changes in the host cells. With newly identified antichlamydial activities and novel study methods we can explore more impactful approaches to overcoming *C. pneumoniae*-induced chronic inflammatory diseases.

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List of original publications

I. Kortesoja, M., Karhu, E., Olafsdottir, E. S., Freysdottir, J., & Hanski, L. (2019). Impact of dibenzocyclooctadiene lignans from *Schisandra chinensis* on the redox status and activation of human innate immune system cells. *Free Radical Biology and Medicine*, *131*, 309-317.

II. Taavitsainen, E., Kortesoja, M., Bruun, T., Johansson, N. G., & Hanski, L. (2020). Assaying *Chlamydia pneumoniae* Persistence in Monocyte-Derived Macrophages Identifies Dibenzocyclooctadiene Lignans as Phenotypic Switchers. *Molecules*, *25*(2), 294.

III. Kortesoja, M., Trofin, R. E., Hanski, L. (2020) A platform for studying the transfer of *Chlamydia pneumoniae* infection from respiratory epithelium to phagocytes. *Journal of Microbiological Methods,* 105857.

IV. Kortesoja, M., Taavitsainen, E., Hanski, L. The influence of dibenzocyclooctadiene lignans on macrophage glutathione and lipid metabolism associated with *Chlamydia pneumoniae*-induced foam cell formation. Manuscript.

In addition, some unpublished data are presented.

Contribution to original publications

I. Designing and performing the experiments conducted with THP-1 cells, data analysis, statistical tests and visualization of the data, writing and revising the manuscript.

II. Designing and performing the experiments concerning ROS and GSH production, data analysis, statistical tests and visualization of the data from the above-mentioned experiments, writing and revising the manuscript in collaboration with another PhD student.

III. Formulating the research questions, designing and performing the experiments and supervising a student who participated in the project, data analysis, statistical tests and visualization of the data gained from all the experiments, writing and revising the manuscript.

IV. Formulating the research questions, designing and performing the experiments, data analysis, statistical tests and visualization of the data gained from all the experiments, writing the manuscript.

Abbreviations

AB	Aberrant body
ABCA1	ATP-binding cassette transporter 1
ACAT1	Acyl-coenzyme A: cholesterol acyltranferase 1
AD	Alzheimer's disease
ApoA1	Apolipoprotein A1
ATCC	American-type culture collection
BSA	Bovine serum albumin
BSO	Buthionine sulfoximine
CAP	Community acquired pneumonia
CASMC	Coronary artery smooth muscle cells
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
COMP	Chlamydial outer membrane complex
COPD	Chronic obstructive pulmonary disease
COX-2	Cyclooxygenase-2
CPAF	Chlamydial protease-like activity factor
CV-6	*C. pneumoniae* cardiovascular strain, CV-6
DCFH-DA	Dichlorodihydrofluorescein diacetate
DCF	Dichlorofluorescein
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EB	Elementary body
EGFR	Epidermal growth factor receptor
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Glutamate cysteine ligase
GGT1	Gamma-glutamyltransferase 1
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione

GSSG	Glutathione disulfide
H_2O_2	hydrogen peroxide
Hct	Histone-like protein
HDL	High density lipoprotein
HL	Human lung (epithelial cell line from human origin)
HLA-A2	Human leukocyte antigen-A2
HMEC-1	Human arterial endothelial cells
HRP	Horse radish peroxidase
Hsp	Heat shock protein
Htra	High temperature requirement A
HUVEC	Human umbilical vein endothelial cells
IFN-γ	Interferon- γ
IFU	Inclusion forming unit
Ig	Immunoglobulin
IKK	I-kappa B kinase
IL	Interleukin
IRAK	IL-1R-assosiated kinase
JNK	c-Jun N-terminal kinase
LAL	Lysosomal acid lipase
LDL	Low density lipoprotein
LOX	Lectin-like oxLDL receptor-1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MIF	Micro-immunofluorescence
MOI	Multiplicity of infection
MOMP	Major outer membrane protein
MyD88	Myeloid differentiation factor
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
NLR	Nucleotide-binding oligomerization domain-like receptor
NLRP3	NLR protein 3
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
Nrf2	Nuclear factor erythroid 2-related factor 2
O_2^-	Superoxide
Omc	Cysteine-rich outer membrane protein

OmpA	Outer membrane protein A
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PMA	Phorbol-12-myristate-13-acetate
PMN	Polymorphonuclear leukocytes
Pmp	Polymorphic membrane protein
PPARγ	Peroxisome proliferator-activated receptor γ
PPR	Pattern recognition receptor
RB	Reticulate body
RIP2	Receptor-interacting-serine/threonine-protein kinase 2
(r)RNA	(ribosomal)Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SOD	Superoxide dismutase
SPG	Sucrose-phosphate-glutamic acid
SR	Scavenger receptor
TAK1	Transforming growth factor-β-activated kinase
Tarp	Translocated actin recruiting phosphoprotein family
TLR	Toll like receptor
TNF-α	Tumor necrosis factor α
TRAF6	Tumor necrosis factor receptor-associated factor 6
TTSS	Type three secretion system
TWAR	*C. pneumoniae* strain
WHO	World Health Organization

1. Introduction

The obligate intracellular bacterium, *Chlamydia pneumoniae* is extremely widely spread in the world [1]. It is a respiratory tract pathogen that is usually treated with antibiotics [2], but its ability to disseminate throughout the body [3] and persist in the organs for a long period of time makes it particularly hard to eradicate. Intracellular persistence, together with the associated inflammatory host responses, have led to the hypothesis of *C. pneumoniae* being a risk factor for numerous severe chronic inflammatory diseases, such as atherosclerosis [4] and Alzheimer's disease (AD) [5]. The current treatment methods are only effective against actively replicating bacteria, and persistent infections may therefore remain in the body. The diagnostics of *C. pneumoniae* infections in the clinic are efficient [6], but the serological markers of the pathogen often appear in a delayed manner. This can in many cases lead to wrong treatment decisions and, further, to the increased recalcitrance of the *C. pneumoniae* infection as a result of an inappropriately administered antimicrobial treatment [7].

C. pneumoniae is able to disseminate within the body in peripheral blood mononuclear cells (PBMCs) and cause several effects inside these cells [3]. Infection in PBMCs induces proinflammatory changes in the cells e.g. inflammatory cytokine production, oxidative stress and lipid accumulation, which promotes the progression of chronic inflammatory diseases, such as atherosclerosis. It would therefore be of great importance to alleviate these infectious actions and eradicate the bacteria from the innate immune system cells [8]. In this thesis, the antichlamydial lead compound profiling is conducted with dibenzocyclooctadiene lignans, isolated from *Schisandra chinensis,* a plant frequently used in traditional Chinese medicine [9].

The inhibition of the transfer of this pathogen from lung epithelium to other cells could also be an effective means to reduce the chronic inflammatory disease progression. We therefore developed a new method for studying the transfer of *C. pneumoniae* from lung epithelial cells to phagocytes. This method could be used in compound profiling, for finding new potential drug candidates against *C. pneumoniae*.

2. Review of the literature

2.1 *Chlamydia pneumoniae*

Chlamydia pneumoniae, a gram-negative bacterium, is a human pathogen whose primary site of infection is the respiratory tract [7, 10]. *C. pneumoniae* is an obligate intracellular bacterium and thus always needs a host cell for its replication and survival. *C. pneumoniae* is a widely spread bacterium and it is estimated that nearly everybody gets infected during their lifetime [1, 11]. A major proportion of the infections remain asymptomatic, but *C. pneumoniae* is able to cause several diseases, such as bronchitis, sinusitis and pneumonia. It is estimated that 10% of all pneumonia cases occurring outside hospitals are caused by *C. pneumoniae.* In addition to respiratory tract infections *C. pneumoniae* has been related to several other diseases, such as atherosclerosis [12, 13], Alzheimer's disease [5, 14] and asthma [15, 16]. To be involved in the progression of chronic inflammatory diseases occurring outside respiratory tract, *C. pneumoniae* must be able to migrate from the respiratory tract to other body sites. This migration occurs via PBMCs, mainly monocytes and macrophages [17, 18].

In addition to actively replicating in the host cells, bacteria may be able to adopt a non-replicative, dormant form that facilitates their prolonged persistence within the cells [7]. *C. pneumoniae* in PBMCs are typically spontaneously switching into such persistent state [8, 19]. Actively replicating bacteria are susceptible to macrolides, tetracyclines and quinolones [2, 20], and azithromycin is the golden standard antibiotic used for *C. pneumoniae* infections [21]. On the contrary, persistent bacteria are refractory to antibiotics and cannot be eradicated effectively [17]. In addition to their increased antimicrobial tolerance, persistent bacteria withstand host defenses for a long period of time, which can lead to chronic infections.

2.1.1 History and taxonomy

Chlamydia pneumoniae has been identified as its own species within the *Chlamydia* genus quite recently, in 1989 [22]. Before it was even recognized as a *Chlamydia*, it was thought to be a virus causing atypical pneumonia [23]. *C. pneumoniae*, previously called TWAR (named after two previously identified isolates, TW-183 and AR-39), were initially isolated by Wang and Greyston from the eye of a child in Taiwan in 1965 [24]. In the beginning it was thought to be a *Chlamydia trachomatis* strain, yet it remained as an unidentified immunotype in 1968 and 1969 when the micro-immunofluorescence (MIF) serologic test for *Chlamydia* was developed. Later, when cell culture methods for *Chlamydia* were developed, TWAR was thought to be a new strain of *Chlamydia psittaci* [25], due to their inclusions sharing similarities and being iodine negative unlike those of *C. trachomatis*. After the MIF test was developed for TWAR antigens, it was realized that this new pathogen was frequently occurring, usually in respiratory infections, and at the end of the 1980s TWAR was recognized as a respiratory pathogen. Unique pear-shaped inclusions and only a 10% DNA homology with *C. trachomatis* and *C. psittaci* [22] led to the identification of TWAR organisms as a new *Chlamydia* species *Chlamydia pneumoniae*, in 1989.

C. pneumoniae species belongs to the phylum of *Chlamydiae,* and class of *Chlamydia*, order of *Chlamydiales* and family of *Chlamydiaceae,* which is divided into two genera: *Chlamydia* and *Chlamydiphila*. *Chlamydia* includes three species: *C. trachomatis, C. suis, C. muridarum* and *Chlamydophila* includes six species: *C. abortus, C psittaci, C. felis, C caviae, C. pecorum* and *C. pneumoniae*. Only *C. trachomatis*, *C. psittaci* and *C. pneumoniae* have been reported to infect humans.

There has been a debate about the nomenclature of the genera and species of *Chlamydia*. In 1999, Everett et al [26], conducted new phylogenetic analyses of the 16S and 23S rRNA genes of *Chlamydiales*. The current taxonomy of the *Chlamydiaceae* family was proposed in which the genera and nomenclature of *Chlamydia pneumoniae* chanced to *Chlamydophila pneumoniae.* As not all scientists have accepted the division of the

species into the genera *Chlamydia* and *Chlamydophila* [27], the naming of *C. pneumoniae* is still controversial and both *Chlamydia pneumoniae* and *Chlamydophila pneumoniae* are used. In this work, only the name *Chlamydia pneumoniae* is used.

2.1.2 Epidemiology and diagnostics

Seroprevalence of *C. pneumoniae* increases with age and it is fairly uncommon with children under the age of 5 [10]. At all ages, *C. pneumoniae* infections are more common in males than in females. Seroprevalence reaches 50% by the age of 20 and by the age of 65 the seroprevalence is more than 70% [11]. *C. pneumoniae* is considered as the most common non-viral intracellular human respiratory pathogen. It is estimated to cause about 10% of community-acquired pneumonia (CAP) and 5% bronchitis and sinusitis cases [10], and the bacterium is frequently found in immunocompromised hosts. According to serological studies, *C. pneumoniae* infection is more frequently occurring than infections with the most studied species of *Chlamydia*, *C. trachomatis* [28], but for example in the Finnish register of infectious diseases there are a lot more *C. trachomatis* cases reported. In 2018, there were 293 reported *C. pneumoniae* cases and 14 839 *C. trachomatis* cases in Finland [29] which means that most *C. pneumoniae* infections are not diagnosed due to their minor or nonexistent symptoms.

Current diagnostic standards include the serological measurement of *C. pneumoniae* antibodies from the blood using an enzyme immunoassay (EIA) [6]. The serological response to *C. pneumoniae* infection differs according to whether the infection is primary or a reinfection. In less than 6 months-old primary infections, both IgM and IgG antibody titers rise, often accompanied with the increase in IgA antibodies. In the case of reinfections, no IgM rise is seen, but IgG and sometimes IgA levels are elevated. It takes at least two weeks for the antibody titers to begin rising after the onset of symptoms and in children the process is even more prolonged. Together with the three to five days required for the completion of the laboratory tests, this delay markedly complicates the selection of antimicrobial treatment.

2.1.3 Structure

C. pneumoniae is classified as a gram-negative bacterium. The cell wall of *Chlamydia* contains two membranes, the outer and inner membrane that are separated from each other by a narrow periplasmic space [1] which has been shown to contain a peptidoglycan layer [30]. There are many cysteine-rich proteins in the chlamydial cell wall that can form disulfide cross-links with each other, which enhances the structural rigidity and osmotic stability of the extracellular bacteria [31, 32]. When bacteria internalize into the host cell, the disulfide bonds are reduced making the intracellular form of *Chlamydia* more fragile [33].

Chlamydia switches between two structurally different forms, elementary bodies (EBs) and reticulate bodies (RBs). EBs are smaller of the two, approximately 0.3 µm in diameter. They are round, spore-like forms with nucleoids highly condensed by bacterial histone-like proteins HctA and HctB [34]. Still, EBs are reported to have a level of metabolism when being outside the cells [20]. RBs are bigger, around 0.8–1 µm in diameter. The nucleoids of RBs are loose and can thus be used for transcription [34, 35].

The outer membrane proteins of *Chlamydia* have been extensively studied. The three most abundant proteins, OmpA, OmcA and OmcB, form the chlamydial outer membrane complex (COMP) [36]. The membrane porin OmpA covers about 60% of all outer membrane protein content and is therefore also known as the major outer membrane protein (MOMP). The oxidation state of the disulfide bridges between the cysteine-rich OmpA proteins takes part in controlling the permeability of the membrane [37]. The proteins OmcA and OmcB form disulfide cross-links with each other, which in turn contributes to the rigidness of the bacteria [36].

The outer membrane of *C. pneumoniae* also includes polymorphic membrane proteins (Pmps). There are 21 genes encoding Pmps, which cover 5% of the *C. pneumoniae* genome [38]. These genes are unique for the *Chlamydiaceae* family, and they have several roles in the chlamydial infection process. Pmps act as adhesins by facilitating the attachment of *C. pneumoniae* to host cells. These proteins have various binding

capabilities depending on the cell type [39]. In addition, Pmps may act as invasins. It is for example reported that Pmp21 binds the EGFR of epithelial cells and initiates the internalization of the bacteria [40]. Targeting the Pmp21 with a specific antibody has been shown to inhibit *C. pneumoniae* infection [41]. Pmp proteins appear to work together with other membrane proteins. For example, Pmp10 expression is reported to protect OmpA from cleavage by proteolytic enzymes [42]. These functions make Pmps important virulence factors in *Chlamydia* and they may provide putative targets for antichlamydial treatment.

Another protein found in the membrane of *C. pneumoniae* is Heat Shock Protein 60 (Hsp60), encoded by the gene *groEL1*. Heat shock proteins are widely expressed within eukaryotic and prokaryotic cells [43]. Hsp60 is synthesized when cells are under stress to act as a chaperone by preventing stress-induced protein misfolding. In addition, it is known to stimulate innate immune system cells, such as macrophages [44]. Hsp60 protein can be recognized by host cells, which makes it a pathogen-associated molecular pattern (PAMP).

2.1.4 Developmental cycle

The chlamydial replication cycle occurs inside a membrane-bound structure called an inclusion [34]. It is formed when the *Chlamydia* becomes surrounded within a host plasma membrane-derived vacuole upon internalization [45]. The inclusion protects the bacteria from the host cell immune system and its fusion with the lysosomes is prevented. Bacteria multiply within the inclusion, whose size increases remarkably as the result. Newly synthetized lipids from the Golgi apparatus are transferred to and incorporated into the inclusion to make its growth possible [45, 46]. On the other hand, only few eukaryotic proteins are reported to be found in the inclusion membrane, and most of the proteins are of bacterial origin [47]. These proteins share a large hydrophobic region and are called the Inc family of proteins. IncA, IncB and IncC are the most abundant proteins in the inclusion membrane, but their functions have not been fully defined [48].

C. pneumoniae has a unique biphasic lifecycle. It contains two morphologically distinct forms: infectious, extracellular EBs and metabolically active, intracellular RBs [34]. When *C. pneumoniae* infection is in the acute replicative state, EBs and RBs are mostly present inside the cells. The EBs occur only in the extracellular space between the replication cycles (Figure 1).

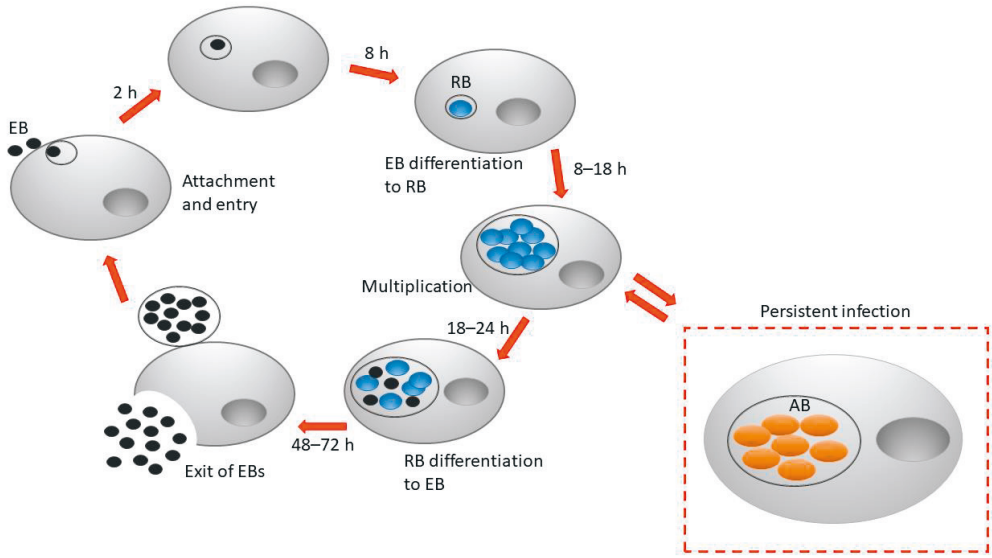


Figure 1. The developmental cycle of *C. pneumoniae*. Elementary bodies (EBs) attach and enter into host cells. After internalization, EBs differentiate into reticulate bodies (RBs) that start to multiply. RBs then re-differentiate into EBs and become released from the cells. RBs can also convert into persistent forms, aberrant bodies (ABs), which are viable but non-replicating forms of the bacterium.

2.1.4.1 Entry

Chlamydial infection starts with the attachment of EB to the host cell. An interaction of *C. pneumoniae* with the host cell occurs in two stages. First, the bacterium attaches reversibly to the host cell, after which an irreversible attachment to a specific receptor occurs. The initial attachment of bacterial adhesins to host cells occurs through heparin

sulfate proteoglycans [49]. This attachment is reversible and happens through electrostatic interactions. Several proteins of the *C. pneumoniae* surface are reported to act as adhesins; OmpA, OmcB, Hsp70 as well as Pmps [49]. After this initial low-affinity binding, host cell receptors bind LPS or the outer membrane proteins of *C. pneumoniae*. Various receptors of human cells can bind *C. pneumoniae* (Table 1). Mannose 6-phosphate receptor [50], epidermal growth factor receptor (EGFR) [40] and apolipoprotein E4 [51], for example, have been suggested to bind *C. pneumoniae* and initiate the internalization. The receptor type and its ligands can vary between the host cell type and the chlamydial strain, respectively [45]. In endothelial cells, insulin-like growth factor 2 receptor and mannose 6-phosphate receptor bind the bacterium [50], while the bacteria utilize the EGFR in epithelial cells for their internalization [40]. It remains unknown which receptors are responsible for the internalization e.g. into immune cells.

Table 1. Proposed receptors of the host cells and ligands of *C. pneumoniae* involved in the internalization of bacteria.

Cell type	Receptor	Ligand	Reference
Human endothelial cells, HMEC-1	Mannoce-6-phosphate receptor / Insulin-like growth factor receptor	ompA	[50]
Human epithelial cells HEp2	Epidermal growth factor receptor	Pmp21	[40]
Human astrocyte cells, U-87 MG	Apolipoprotein E4	Unknown	[51]
Human epithelial cells, HEp2	Unknown	Pmp6, Pmp20	[38]

MOMP, major outer membrane protein; Pmp, Polymorphic membrane protein.

Immediately after receptor binding, chlamydial type three secretion system (TTSS) proteins are injected into the host cell to make the environment more suitable for the bacteria [52]. The TTSS consists of approximately 25 proteins [53], including

chaperones and ATPases, which are highly conserved across the organisms possessing this injection machinery [54, 55]. The Inc proteins of chlamydial inclusions are examples of TTSS effectors [48]. Another protein secreted by the TTSS is the translocated actin recruiting phosphoprotein family (Tarp) [56]. The chlamydial internalization into the host cells requires actin cytoskeleton remodeling at the site of internalization. Tarp is a protein that recruits actin at the entry site by directly interacting with it and promoting its nucleation, which is an initial and rate-limiting step in actin polymerization [57]. Studies also report the possible participation of vinculin, a cytoskeletal protein, which is involved in anchoring actin to a cell membrane [58]. Tarp colocalizes with vinculin and recruits it within the polymerized actin. In addition to actin remodeling, massive membrane remodeling occurs during the internalization of *Chlamydia* [59].

2.1.4.2 Differentiation and multiplication

After internalization, the bacterial proteins are organized on the inclusion membrane, which enables the inclusion to evade the endosomal pathway and lysosomal fusion [59]. The inclusion then migrates near to the peri Golgi region, which is a nutrient rich region and thus advantageous to the bacteria [34, 45]. Within the inclusion, EBs quickly begin to differentiate into RBs [1]. The very dense, condensed DNA is loosened into a more fibrillar form and becomes transcriptionally active almost immediately after internalization. Proteins involved in nutrient acquisition, inclusion modification and transcription regulation are synthetized and more ribosomes are formed to enable effective protein synthesis [55]. The cell wall of the bacteria becomes more flexible and thinner, which makes it more fragile. RBs multiply by binary fission, in which DNA replicates and the cell is divided into two equally-sized daughter cells [60]. During the multiplication of RBs the inclusion expands, and at the end of the replication cycle it covers a large region of the host cell's cytoplasm. Chlamydial proteases, such as chlamydial proteasome-like activity factor (CPAF) and high temperature requirement A (HtrA), are virulence factors that break up host cell proteins and cause several effects on the infected host cells [61]. They have an essential role in the chlamydial replication

cycle progression during this replication phase. RBs differentiate back to EBs asynchronously. During this secondary differentiation, proteins of the outer membrane protein complex (OmpA, OmcA, OmcB), are cross-linked in the outer membrane and the chromosome condensation genes (histone-like genes) become expressed [55]. At 48 to 72 h post infection, newly formed EBs are released from the host cells.

2.1.4.3 Exit

At the end of the chlamydial replication cycle, approximately 48–72 hours after the infection, new EBs are released from the host cell by two distinct mechanisms: by host cell lysis or by the extrusion pathway [62]. In the lysis exit, the inclusion membrane first dissociates, followed by the breakdown of the host plasma membrane, and free EBs become released to the extracellular space [62]. In extrusion, the whole inclusion pinches out from the cell leaving the host cell intact. In this case, EBs are left inside the membrane-bound vacuole in the extracellular space. An inclusion can also be released from the cell only partly leaving some of the bacteria inside the host. The extrusion vacuole is suggested to protect EBs in the extracellular space from phagolysosomal killing [63], and EBs in extrusions have been shown to survive better in cell culture compared to free EBs. The extrusion vacuoles can also be internalized as such into phagocytes in which they can disseminate the infection, so exit by extrusion seems to be beneficial for chlamydial survival also in this regard. It remains unknown whether *C. pneumoniae* can be released from the cells via both exit approaches. As opposed to other *Chlamydia* species, there is no experimental data available of the capability of *C. pneumoniae* to exit by host cell lysis. Instead, extrusion has been demonstrated also in *C. pneumoniae* [64].

2.1.5 Persistent infections

If, during the active development, *Chlamydia* experiences stressful conditions, such as nutrient deprivation or antibiotic treatment, its replication cycle may be interrupted [7]. This prompts *Chlamydia* to enter an inactive state in which they can withstand such conditions for prolonged times. Also known as persistence, this chlamydial stress response is transient, and the replication cycle can revert into the normal state upon the re-establishment of favorable conditions.

The morphologically distinct persistent bacteria are called aberrant bodies (ABs) or aberrant RBs [34]. ABs can be identified by electron microscopy based on their enlarged size and irregular shape. Chlamydial persistence can be induced by various stimuli *in vitro.* Exposure to the penicillin group of antibiotics [65], nutrient deprivation (e.g. iron, amino acids, glucose) [66], interferon-γ (IFN-γ) exposure [67], tobacco smoke [68] among other stress inducers have been reported to initiate persistence. Penicillin blocks the RB multiplication and redifferentiation into EBs [7]. IFN-γ is a cytokine that induces the immune system to fight the pathogens and it also increases the catabolism of tryptophan [69], which is an essential amino acid for *Chlamydia*.

When *Chlamydia* evades the innate immune responses of the cells and enters persistence, RB-to-EB conversion is blocked and cell division stops. Gene expression is also altered. The genes involved in RB-to-EB differentiation, such as *omcB* and *hctB*, are not expressed and stress response genes, such as heat shock proteins *grpE*, *groES* and *htrA*, are overexpressed instead to protect the bacteria in stressful conditions [70].

In addition to *in vitro* persistence models, *Chlamydia* can also enter the persistent state *in vivo*. With immunostaining, aberrant bodies from different *Chlamydia* species have been observed in various tissues and chlamydial DNA has been found in tissues long after the obvious infection [7]. In several cases the bacteria are in a non-cultivable state when isolated, which indicates a persistent infection. A key outcome of *C. pneumoniae* persistence is reoccurring infections. It is hypothesized that during *Chlamydia* infection, a subpopulation of bacteria enters into a persistent state due to stressful conditions, while

others continue to replicate normally. Replicating bacteria can eventually be eradicated by the host immune system or by antibiotic treatment, while persistent bacteria remain and continue to manipulate their microenvironment to their favor, generating a chronic and long-term infection with minimal symptoms. According to murine models, it seems that immune reactions can induce *C. pneumoniae* persistence, and when the stressful conditions have subsided, the bacteria can be reactivated in the tissues [71]. This provides further evidence to the notion that persistence is the main driver behind reoccurring *C. pneumoniae* infection. A similar phenomenon has been identified also in other intracellular bacteria. For example, *Staphylococcus aureus* and *Escherichia coli* have been reported to enter the persistent state inside host cells under stressful conditions [72, 73].

2.2 *Chlamydia pneumoniae* in innate immune system cells

The primary site of infection of *C. pneumoniae* is the respiratory tract and in there, the epithelial cells [74]. In addition, *C. pneumoniae* can infect various other cell types, among which the most studied are the innate immune system cells, including peripheral blood mononuclear cells: monocytes and macrophages [8, 75, 76]. *C. pneumoniae* has been shown to change the normal functions of these cells in several ways. These changes are associated with chronic inflammatory diseases, especially the progression of atherosclerosis [77]. For example, the bacterium induces inflammatory cytokine production, alters the redox balance and induces foam cell formation of macrophages, which are particularly important in atherosclerosis development [8].

2.2.1 Dissemination in the body

C. pneumoniae has been found in various tissues throughout the body. The transport of *C. pneumoniae* from the respiratory tract to other body sites occurs via PBMCs,

including mainly monocytes that can differentiate into macrophages when entering their target tissue [3]. The dissemination has been studied with murine models [18, 78]. When mice were inoculated intranasally or intraperitoneally, the bacteria could be found in alveolar macrophages and peritoneal macrophages, respectively, and also in monocytes. When infected macrophages were injected into uninfected mice, the bacteria were found in lungs, lymph nodes, the thymus and spleen. Rabbit models have also been used in dissemination studies [3]. Rabbits were infected intratracheally and after the inoculation bacteria could be detected from granulocytes, alveolar epithelium and alveolar macrophages. The alveolar macrophages transferred the bacteria into lymph nodes and from there *C. pneumoniae* was disseminated within PBMCs into the spleen and aorta. *C. pneumoniae* DNA has been found in PBMCs of patients with cardiovascular disease [79] and viable bacteria have also been extracted from atherosclerotic arteries [80, 81]. One mechanism that *C. pneumoniae* employs to transfer into macrophages is silent entry through polymorphonuclear leucocytes (PMN) [82]. When *C. pneumoniae* infects the respiratory tract, PMNs are recruited to the infection site and the bacteria may infect them. Infected PMNs recruit monocytes to the infection site and, when the infection leads to the apoptosis of PMNs, the monocytes phagocytize them. This mechanism is suggested to support *C. pneumoniae* internalization into monocytes.

2.2.2 Infection in monocytes and macrophages

2.2.2.1 Inflammatory cytokine production

Cytokines are small proteins secreted by various types of cells, including immune system cells [83]. They act as signal molecules and immunomodulatory agents. *C. pneumoniae* infection induces both, pro- and anti-inflammatory cytokine production, in immune system cells. For example, infection has been shown to induce the production of tumor necrosis factor α (TNF-α), IL-1β and IL-6 in several cell lines. *C. pneumoniae*-induced cytokine production in different cell lines is reviewed in Table 2.

Table 2. The cytokines induced by *C. pneumoniae* in different cell types of innate immune system cells.

Cell line	Organism	cytokine	Reference
Mono Mac 6	human	TNF-α, IL-1β, IL-6	[84]
DCs	mouse	TNF-α, IL-12p40	[44, 85]
RAW264.7	mouse	TNF-α, IL-1β, IL-6, MCP-1	[86]
HPBMCs	human	TNF-α, IL-1β, IL-6, IFN-α, IL-8, IL-10, MCP-1	[87-89]
AM	human	TNF-α, IL-1β, IL-8	[90]
THP-1	human	TNF-α, IL-1β, IL-8, IL-12, IL-10	[91-93]

AM, alveolar macrophages; HPBMC, Human peripheral blood mononuclear cells; DC, dendritic cells; IFN-α, interferon α; IL, interleukin; MCP, Monocyte chemoattractant protein-1; TNF-α, tumor necrosis factor α.

2.2.2.2 Redox balance alteration

In addition to cytokines, innate immune system cells produce also other types of regulators in response bacterial infection. These include reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-), and reactive nitrogen species (RNS), such as nitric oxide (NO) [94]. ROS are produced in endothelial cells and macrophages through NADPH oxidase (NOX), as well as through the mitochondrial respiratory electron transport chain. An excessive amount of ROS in the cells induces oxidative stress which promotes the progression of inflammatory diseases. *C. pneumoniae* has been reported to alter the redox balance of infected cells [95], e.g. by inducing the ROS and NO production [96, 97]. In murine macrophages, *C. pneumoniae* has been reported to induce both the NOX-induced and mitochondrial ROS production [98]. Although this ROS production is a defense mechanism against *C. pneumoniae* it has been reported that the pathogen is able to survive high ROS levels by concomitantly altering the antioxidant levels of the cells [99]. In addition, *C. pneumoniae* has been reported to induce NO production in murine macrophages [97]. NO is synthetized by inducible nitric oxide synthase (iNOS), and *C. pneumoniae* is suggested to induce NO production through altering iNOS expression [100, 101].

While *C. pneumoniae* infection alters the redox state of the host cells, it is also reported that the alteration may in turn affect the *C. pneumoniae* infection phenotype in mouse macrophages [99, 102]. The suppression of oxidative stress by antioxidant supplementation increased the inclusion formation in the cells, and when the redox status was increased, the inclusion formation was correspondingly decreased. It is suggested that *C. pneumoniae* switches into a more persistent state in the intracellular oxidative environment, which leads to the reduced inclusion formation.

Alleviating the oxidative stress caused by *C. pneumoniae* infection has been considered as a potential treatment option in the prevention of *C. pneumoniae*-induced atherosclerosis [95]. Diet-derived antioxidants, vitamin E, resveratrol and curcumin as well as COX2 inhibitors have been shown to prevent *C. pneumoniae*-induced ROS production in different cell lines.

2.2.2.3 Glutathione metabolism

Glutathione (γ-glutamyl-cysteinyl-glycine, GSH) is a major antioxidant in cells [103-105]. It is a tripeptide that is present in all tissues throughout the body. It efficiently scavenges ROS and other free radicals, including hydroxyl radicals, lipid peroxyl radicals and H_2O_2. The scavenging can occur directly or through enzymatic reactions, in which GSH is oxidized into glutathione disulfide (GSSG). GSSG is then converted back to GSH by reductases in the presence of NADPH. GSH prevents the oxidation of biomolecules in the cells and it also reacts with several biological metabolites and xenobiotics, i.e. foreign chemical or biological substances. In addition to the scavenging activity, GSH also has a more general role in metabolism. Among other functions, it takes part in prostaglandin and leukotriene synthesis and transports and stores cysteine. In addition, GSH has several regulatory functions involved in intracellular redox status, gene expression, DNA and protein synthesis, signal transduction, the proliferation and apoptosis of the cells as well as mitochondrial function.

GSH is synthetized in the cytosol, after which it may be transferred into other cellular compartments [106]. A part of the GSH pool remains constantly in the cytosol, but the rest is distributed into the endoplasmic reticulum (ER), nucleus and mitochondria. GSH is also distributed between different organ systems, particularly liver, kidneys and pancreas, where GSH is needed to detoxify xenobiotics [107-109].

The cellular GSH balance is maintained between consumption and synthesis [106]. Due to their strong association with cellular oxidative stress, altered GSH levels have been associated with several pathologies, including cardiovascular [110] and neurogenerative diseases [111], cancer [112] and diabetes [113].

C. pneumoniae infection has been reported to alter cellular GSH levels [114]. For instance, it was demonstrated to suppress the GSH pools in human T cells and also in murine RAW264.7 macrophages [99]. On the other hand, GSH supplementation demonstrated to increase *C. pneumoniae* replication in RAW264.7 cells, which also links GSH metabolism to *C. pneumoniae* infection and survival [102]. This is also associated with the chlamydial infection phenotype and the induction of persistence, as described above.

C. pneumoniae has also been reported to affect the antioxidative enzyme production in murine macrophages [99]. It induced the ROS-scavenging superoxide dismutase (SOD) and glutathione peroxidase (GPx) even after being heat-inactivated, which implies that bacterial replication is not necessary for the effect. Infection additionally induced the γ-cysteine ligase (GCL) production, which catalyzes glutathione biosynthesis in the macrophages. These may be compensatory mechanisms arising from the *C. pneumoniae*-induced ROS/NO production and the decreased levels of GSH.

2.2.2.4 Foam cell formation

Foam cell formation is one of the first hallmarks of atherosclerosis [115]. Foam cells are macrophages in which lipids are accumulated into vacuoles, giving the cells a foamy

appearance. Foam cells accumulate in arterial walls and initiate the formation of an atheroma [116]. Several factors promote foam cell formation, including inflammatory cytokines, transcription factors, a lipid-rich environment, and oxidative stress. In oxidative environment, LDL is oxidized and is thus more likely to accumulate in the cells [117].

Scavenger receptors are responsible for recognizing and internalizing the oxidized LDL into the cells [94]. There are ten different classes of scavenger receptors, SR-A to -J [118]. The ones most related to foam cell formation are SR-A and SR-B. There are several reports of the cluster of differentiation 36 protein (CD36), which belongs to SR-B family, being involved in macrophage foam cell formation. It is suggested that 60–70% of macrophage foam cell formation is CD36-dependent [119, 120]. CD36 is an integral membrane protein found in many cell types. Native, unmodified LDL internalizes into the cells by different mechanisms than oxidized LDL. One such mechanism is through fluid-phase pinocytosis, which is an endocytic pathway without any specific receptor binding [121, 122].

After LDL is internalized, lipoproteins are delivered to the late endosome where they are broken up to cholesterol and fatty acids [123]. The esterification of the free cholesterol is another phenomenon that takes place in the macrophage foam cells [124]. Cholesteryl esters are more likely to accumulate into lipid vacuoles and promote foam cell formation [125]. The free cholesterol inside the cells is esterified by acyl-coenzyme A cholesterol acyltranferase 1 (ACAT1) [124]. Cholesteryl esters are simultaneously hydrolyzed back to free cholesterol by lysosomal acid lipase (LAL) and the rate of cholesterol accumulation into lipid droplets is determined by the balance of esterification and hydrolysis.

Another important factor concerning lipid accumulation and foam cell formation is lipid efflux from the cells. Lipid accumulation itself induces efflux, which occurs mostly via the ATP-binding cassette (ABC) transporters ABCA1 and ABCG1 [126]. ABCA1 transports cholesterol from macrophages to apolipoprotein A1 (ApoA1). ApoA1 is a

major component of HDL particles that transfer lipids out of the cells. The ABCA1 on the macrophage surface forms a complex with ApoA1, connecting the HDL particle to the cell, and then transports cholesterol from the cell to the HDL. A summary of lipid transportation in cells is presented in Figure 2.

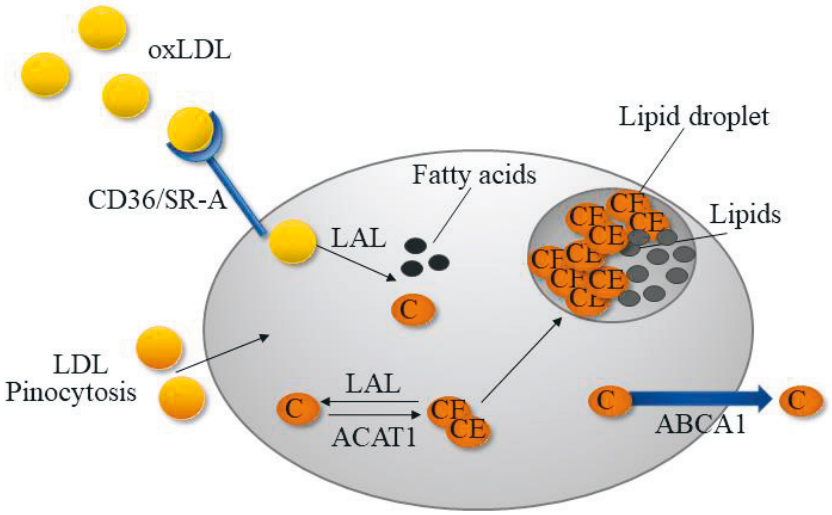


Figure 2. Cholesterol traffic inside the macrophage foam cells. Oxidized LDL is taken into the cells by scavenger receptors i.e. CD36 and SR-A. Unmodified LDL, in turn, is taken into the cells by pinocytosis. LAL breaks LDL into fatty acids and pure cholesterol (C), which can be esterified by ACAT1. Oxidized LDL, cholesteryl esters (CE) and other lipids accumulate into lipid droplets, which are formed inside the foam cells. Lipids are transported out of the cells via the efflux protein ABCA1.

Peroxisome proliferator-activated receptor gamma (PPARγ) activity plays an important role in several events related to lipid accumulation and foam cell formation. PPARγ is a nuclear receptor that regulates the expression of the genes involved in lipid and glucose metabolism [127], such as *CD36* [128] and *ACAT1* [129]. PPARγ also takes part in inflammatory response e.g. by inhibiting the nuclear factor kappa B (NF-κB) activation [130]

C. pneumoniae has been associated with macrophage foam cell formation and atheroma development in human and murine cell lines [131-134]. The Hsp60 of *C. pneumoniae* is reported to increase LDL oxidation [135, 136]. Infection has been also shown to increase the amount of cholesterol esters in human macrophages [131] as well as in murine macrophages, in which foam cell formation was shown to be induced by chlamydial LPS [137]. The increase in cholesterol was revealed to occur via ACAT1 activation in THP-1 macrophages [138]. It has also been reported that *C. pneumoniae* reduced the ABCA1-related cholesterol efflux [139] and PPARγ activity [140] in THP-1 macrophages.

2.2.2.5 Cell signaling pathways

C. pneumoniae is recognized by immune cells though pattern recognition receptors (PPRs), toll like receptors (TLRs), and nucleotide-binding oligomerization domain-like receptors (NLRs). TLRs localize in the cell membrane and recognize extracellular *C. pneumoniae*. NLRs are intracellular receptors and recognize the bacteria inside the cells. Several studies attempted to identify the TLRs responsible for this recognition, and both TLR2 and TLR4 have been proposed [141]. TLR activation leads to several responses in the cells via the transcription factors NF-κB, which regulates the expression of genes involved in survival, immune responses and inflammation [142]; and PPARγ, which plays an important role in lipid metabolism in the cells [134].

TLR activation leads to the induction of myeloid differentiation primary response 88 protein (MyD88), an adaptor that relays the outside signals through TLR to the intracellular signaling system by linking them with the IL-1R-assosiated kinase (IRAK) [143]. Another adaptor protein activated by TLRs, the TNF receptor-associated factor 6 (TRAF6), then activates the MAPK kinase pathways c-Jun NH2-terminal kinase (JNK) pathway and p38 mitogen-activated protein kinase pathway [144], resulting in the regulation of PPARγ. TRAF6 binds a complex including transforming growth factor-β-activated kinase (TAK1), which in turn activates I-kappa B kinase (IKK) complex, finally leading to the activation of nuclear factor-kappa B (NF-κB) [145]. Intracellular

NLRs activate receptor-interacting-serine/threonine-protein kinase 2 (RIP2) which also induces MAPK signaling and NF-κB activation [146].

C. pneumoniae has been reported to activate the MAPK signaling pathway. Chlamydial antigens were discovered to activate the JNK pathway in murine [147] and human [134] macrophages. Furthermore, *C. pneumoniae* infection has been reported to affect NF-κB [139, 148] and PPARγ [149] pathways. A non-exhaustive presentation of the signaling pathways induced by *C. pneumoniae* are presented in Figure 3.

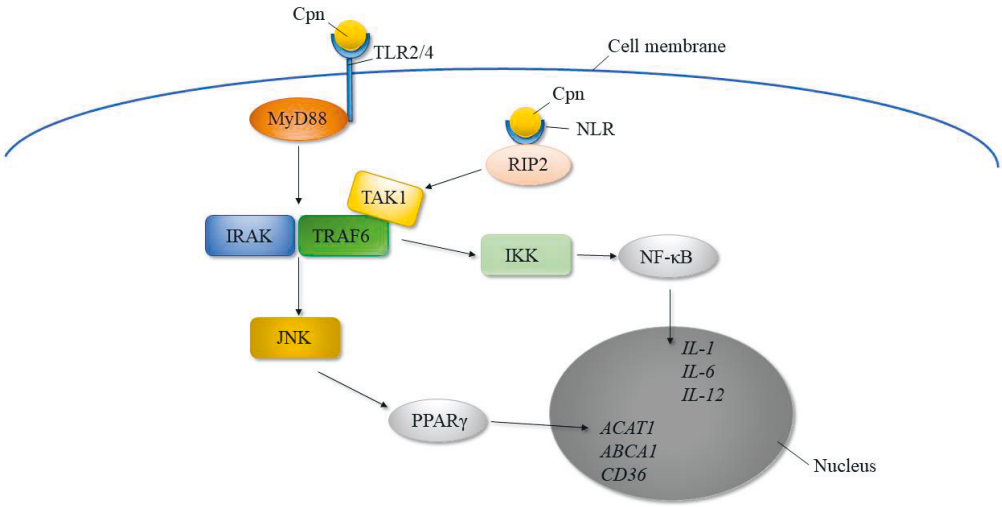


Figure 3. *C. pneumoniae*-induced cell signaling pathways related to inflammation and lipid metabolism. *C. pneumoniae* (Cpn) is recognized by TLRs and NLRs in immune cells and it causes immune reactions by activating the NF-κB transcription factor. The PPARγ transcription factor is also induced, which affects lipid metabolism in the cells.

2.2.3 Links to chronic inflammatory diseases

2.2.3.1 Atherosclerosis

The association of *C. pneumoniae* with atherosclerosis dates back for decades. *C. pneumoniae* was first discovered in an atherosclerotic plaque by Shor et al in 1992, who used transmission electron microscopy, immunohistochemistry, and PCR [150]. This was followed by several other discoveries of *C. pneumoniae,* including viable bacteria, in atherosclerotic lesions [12, 80, 151, 152]. The cultivation of *C. pneumoniae* after extracting it from atheromatous tissues has been difficult, the most likely reason being that bacteria are in their non-replicative persistent state.

Several seroepidemiological studies have been carried out to characterize the association between atherosclerosis and *C. pneumoniae*. The first study was conducted by Saikku et al. in 1988 [153] followed by several others [13, 154-157], most of them finding a significant serological association between the disease and *C. pneumoniae* infection.

Murine and rabbit models have provided more evidence of the association between *C. pneumoniae* and atherosclerosis development, since *C. pneumoniae* infection increased the atheroma formation in both models, and viable bacteria could be isolated from the lesions afterwards [158, 159].

The mechanisms by which *C. pneumoniae* promotes atherosclerosis progression have been studied widely, and there is evidence that *C. pneumoniae* might have a role in all stages of the disease [13]. Inflammation within the arterial wall is considered to be the initial event in atherosclerosis progression and *C. pneumoniae* may take part in that by infecting the vascular endothelial cells, which leads to inflammatory cytokine production as well as the migration and adhesion of leucocytes [160]. In addition to foam cell formation, *C. pneumoniae* may contribute to the latter stages of atheroma development. Infected endothelial cells secrete growth factors which, together with cytokines from macrophages and oxidized LDL, can promote smooth muscle cell proliferation and migration [161]. The smooth muscle cells in turn secrete molecules that promote the

atheroma formation. Matrix metalloproteases (MMPs) are known to weaken the atherosclerosis plaques and promote their rupture [162]. Chlamydial Hsp60 stimulates MMP production in atheroma and can thus be additionally involved in atheroma rupture [163].

2.2.3.2 Alzheimer's disease

Because of a previously-established connection between microorganisms and neurologic disorders, researchers have investigated the association of *C. pneumoniae* with Alzheimer's disease (AD) for a long time. Balin et al, in 1998, demonstrated with immunological labeling and PCR that *C. pneumoniae* antigens and DNA were present in the areas in the brain showing neuropathology in AD patients [164]. Several subsequent trials attempted to repeat these results and provide further evidence for the relationship between the pathogen and AD, but these attempts were unsuccessful [165-167]. Later on, several studies have discovered *C. pneumoniae* in the brain tissue of AD patients, thus strengthening the link between *C. pneumoniae* and AD [168-170]. Similar associations have also been demonstrated in animal models [14]. One suggested mechanism by which *C. pneumoniae* affects the brain is connected to the ApoE-ε4 allele. ApoE is a major apolipoprotein in the brain, and its variant, produced from the allele ε4, is associated with a higher risk of Alzheimer's disease [171]. Patients with the ε4 allele have also shown to contain increased amount of *C. pneumoniae* infected cells. This isoform of ApoE4 also seems to enhance the attachment of *C. pneumoniae* to the cells [172].

2.2.3.3 Obstructive lung disease

C. pneumoniae has been related to both the development and severity of asthma [173, 174]. It can infect alveolar macrophages and lung monocytes, which causes inflammatory cytokine production that is common in allergic and asthma patients [175].

Such infection also causes immunopathologic damage to lung tissue. *C. pneumoniae*-specific antibodies have also been found in a majority of patients with severe asthma, compared to the samples from healthy donors [174]. Azithromycin, the standard antibiotic used in *C. pneumoniae* infections, has been found to alleviate asthmatic symptoms and improve the quality of life of the patients, which further points towards the role of infectious processes in chronic respiratory illnesses [176]. However, azithromycin itself has immunomodulatory effects, which could have contributed to the positive outcomes.

There is also evidence that *C. pneumoniae* infection is associated with chronic obstructive pulmonary disease (COPD). Bacteria have been found in bronchioli, alveoli and alveolar macrophages extracted from COPD patients [177].

2.3 Antichlamydial drug discovery and lead compound profiling

2.3.2 Current treatment

Antibiotics that interfere with DNA and protein synthesis are used for chlamydial infections. Tetracyclines (tetracycline, doxycycline), macrolides (azithromycin) and quinolones are most commonly used [2].

Azithromycin and other macrolides are the most commonly used antibiotics for *C. pneumoniae* infections. Their mechanism of action is to bind the bacterial ribosome S50 subunit and prevent peptidyl-tRNA binding to the P-site of the ribosome, which leads to the inhibition of protein synthesis in bacteria [178]. Tetracyclines also target microbial protein synthesis, but they do so by binding the 30S subunit of the ribosome, thus inhibiting the binding of aminoacyl-tRNA to the A-site of the ribosome. Quinolones interfere with the unwinding and replication of bacterial DNA. Regardless of the treatment standards for *C. pneumoniae*, the delays in diagnosis often lead to non-optimal antibiotic treatment, as is the case with all persistent bacteria [179]. In Finland, the

standard procedure is to start antibiotics immediately when pneumonia is diagnosed, or even before if symptoms are strongly indicating it [180]. Because the most common causative agent of community acquired pneumonia is *Streptococcus pneumoniae*, the standard antibiotic of choice is amoxicillin, a beta-lactam antibiotic [181]. It has been reported, however, that beta-lactam antibiotics, such as penicillin, ampicillin, amoxicillin and carbenicillin induce chlamydial persistence *in vitro* [7, 65, 182]. Therefore, not only can beta-lactam antibiotic treatment for pneumonia be ineffective due to the non-susceptible pathogen, but it may also promote the development of a persistent *C. pneumoniae* infection.

2.3.3 Natural products as lead compounds

Several approaches have been applied for the discovery of novel antichlamydial compounds. Existing drugs such as statins [183, 184] and heparins [185] have been studied for this purpose, but new investigational compounds have been proposed, including 2-arylbenzimidazoles [186] and $\beta^{2,2}$-aminoacid derivatives [31]. An alternative approach has been to explore natural compounds. Natural products have been used as herbal medication since the ancient times, but the drug discovery of purified compounds from natural products has only been ongoing since the 19th century [187]. It is a challenging and time-consuming approach for drug discovery, because it includes the identification and phytochemical as well as pharmacological characterization of bioactive compounds [188]. Still, there have been many successful discoveries from natural products, common examples being acetylsalicylic acid, an anti-inflammatory drug originally derived from the willow plant (*Salix alba*) [189]; quinine, an antimalarial drug from the bark of the cinchona tree (*Cinchona officialis*) [190]; opioid pain killers from opium poppy (*Papaver somniferum*) [191]; digoxin, a cardiac medicine, from common foxglove (*Digitalis purpurea*) [192], and paclitaxel, an anticancer drug from the bark of the Pacific yew tree (*Taxus brevifolia*) [193].

According to estimates [188], there are 75 000 species of higher plants of witch only 10% have been used in traditional medicine, and only 1 to 5% have been studied scientifically. Exploring the active compounds of products that have been used as traditional medicine has been proven a fruitful approach in drug discovery, providing many possibilities for finding new effective and safe lead compounds.

During the past few decades, the efficacy of the available antimicrobials has been in steep decline due to the rapid emergence of antibiotic resistance in pathogens. This poses a serious threat for healthcare. According to the World Health Organization (WHO), we are heading towards 'a postantibiotic era', in which minor injuries and common bacterial infections can kill people, because there are no efficient antibiotics left [194]. Even though approximately two thirds of current antimicrobials are derived from the nature [195], pharmaceutical companies have very little research interest in naturally derived compounds. The most challenging tasks in natural product-based drug discovery are the target deconvolution, the active compound identification and the synthesis of the often very complicated structures. Several natural products, especially phenolic compounds, have been shown to have antichlamydial activity (Table 3). Therefore, the exploration of natural products appears to be an effective approach for antichlamydial drug discovery.

Table 3. Examples of naturally occurring phenolic compounds with activity against *C. pneumoniae*.

Compound group	Example compounds	Reference
Flavones	Luteolin	[196-198]
Flavonols	Quercetin, rhamnetin	[196-199]
Flavanones	Naringin	[196]
Isoflavones	Biochanin A, genistein, rotenone	[196, 200, 201]
Coumarins	Coumarin	[196, 202]
Catechins	Catechin, epigallocatechin gallate	[196, 202]
Phenolic acids	Caffeic acid, rosmarinic acid	[196, 197]
Stilbenes	Resveratrol	[196]
Lignans	Schisandrin B, schisandrin C	[203]

2.3.4 Future prospective

It is estimated that bringing new drug to the market takes 10 to 15 years and costs billions of dollars [204]. The methodology in antichlamydial drug discovery and lead profiling studies differs from that of several other pathogens. Since *Chlamydia* is an obligate intracellular bacterium and not viable outside cells, infecting mammalian cells is a crucial step in the experiments. This makes the drug discovery considerably more time-consuming when compared to typical antimicrobial research workflows. Permissive cell lines are widely used in the drug development studies, and the detection of chlamydial replication is a standard method in *Chlamydia*-research [205, 206]. This approach leaves the non-replicative, persistent infections unnoticed. Considering that persistent infections occur spontaneously in several cell lines, including the ones used in the study of chronic inflammatory diseases, this limitation is critical. Thus, in addition to new potential antichlamydial drugs, new methods for antichlamydial lead profiling are also

needed to enhance the discovery of effective drugs against active and persistent *C. pneumoniae* infections.

2.4 Lignans from *Schisandra chinensis*

Schisandraceae is a family of flowering plants that belongs to the order of *Austrobaileyales* [207]. *Schisandraceae* are divided into three genera: *Kadsura, Illicium* and *Schisandra*. Apart from a few exceptions, all of the approximately 90 species of *Schisandraceae* are distributed in southeastern Asia, mainly in China, and they have been widely used in traditional Chinese medicine. *Schisandra chinensis*, more commonly known as the Chinese magnolia-vine, is one of the most used medicinal plants among *Schisandraceae* [9]. *S. chinensis* produces red berries that grow in hanging clusters, and these berries are the parts used for herbal remedies [208]. In addition to China, *S. chinensis* berry extract has also been used in the western countries. Its traditional applications have included the treatment of GI- and respiratory tract diseases, cardiovascular diseases, fatigue and insomnia [9].

Lignans are polyphenols, frequently occurring metabolites that are found in various plants. Their structure includes two or more phenylpropanoid units [209]. Dibenzocyclooctadiene lignans occur in several species of the genera *Schisandra* [210] and *Kadsura* [211]. In addition to two phenyl rings, dibenzocyclooctadiene lignans contain an eight-ring structure, which makes them unusual among polyphenols [209]. Dibenzocyclooctadiene lignans are dominant secondary metabolites in the *S. chinensis* berries and they have been found to have many biological activities [9, 208, 212]. Approximately 150 lignans with the dibenzocyclooctadiene structure have been discovered [213] and in most part in *S. chinensis,* which is the reason these molecules are often referred to as *S. chinensis* lignans [214]. The most abundant dibenzocyclooctadiene lignans in the berries are schisandrin, schisandrin B, schisanterin A and schisanterin B.

Schisandrin lignans have been widely studied, but the mechanisms of action of these compounds are only partially understood. They have been reported to have cardio- [215], neuro- [216] and hepatoprotective [217] effects and they have also demonstrated anticancer properties by preventing metastasis formation in the murine breast cancer models [218]. They act as immunomodulatory agents by reducing LPS-induced cytokine production [219, 220] as well as the production of ROS and RNS [221]. The antioxidative properties of schisandrin lignans have been shown to be linked to the glutathione homeostasis in various cell types [217, 220, 222], but there is a limited amount of data available on immune cells and none on monocytes and macrophages. Very few reports have explored the putative antimicrobial activity of schisandrin lignans or the berry extract, but previous studies conducted in our laboratory introduce the antichlamydial activity of single dibenzocyclooctadiene lignans [203].

There is a limited amount of data available about the absorption, distribution, metabolism and excretion properties of schisandrin lignans. A few bioavailability studies have been conducted in mice for schisandrin and schisandrin B, and it seems that oral administration shows a bioavailability of up to 50% [223, 224].

3. Aims of the study

The *C. pneumoniae* infection in monocytes and macrophages is a major problem since the infection changes the normal function of these cells and the pathogen switches into a persistent form. Susceptibility screenings of potential antichlamydial compounds are typically done in permissive cell lines, in which the persistent infection remains unnoticed. The aims of this thesis focused on establishing a workflow for the evaluation of potential antichlamydial compounds on their ability to suppress *C. pneumoniae*-induced pathologies in monocytes and macrophages. The objectives also included the profiling of antichlamydial lead compounds, dibenzocyclooctadiene lignans, in *C. pneumoniae*-infected monocyte-macrophage cell models.

The specific aims of the work were:

- To study the impact of schisandrin lignans on the redox status of a human monocytic cell line and on the activation of human innate immune system cells (I)
- To study the impact of schisandrin lignans on the redox status in macrophage cell lines and its effect on the *C. pneumoniae* replication status (II, IV)
- To study the impact of schisandrin lignans on foam cell formation and its connection to gene expression related to lipid accumulation and glutathione metabolism (IV)
- To examine the transfer of *C. pneumoniae* from epithelial cells to phagocytes and evaluate the possible mechanisms to inhibit the transfer (III)

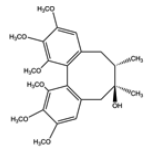
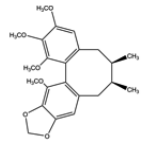
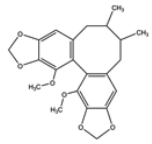
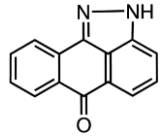
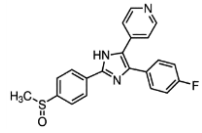
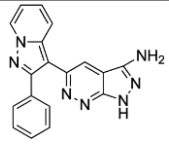
4. Materials and methods

4.1 Studied compounds

The main compounds used in the studies are three dibenzocyclooctadiene lignans, originally isolated from the berries of *Schisandra chinensis* (Table 4). Schisandrin was purchased from Sigma-Aldrich (St. Louis, MO, USA) and schisandrin B and schisandrin C from Fine Tech Industries (London, UK). The compounds were dissolved in dimethyl sulfoxide (DMSO) yielding a concentration of 20 mM and they were stored at -20 °C. The lignans were used in experiments at the final concentrations of 25 µM and 50 µM.

Three MAPK inhibitors, c-Jun N-terminal kinase (JNK) inhibitor (SP600125), p38 mitogen activated protein kinase inhibitor (SB203580) and extracellular signal-regulated kinase (ERK) inhibitor (FR180204), were used in the studies. They were purchased from Tocris Bioscience (Bristol, UK) and dissolved in DMSO at a final concentration of 20 mM. The MAPK inhibitors were used in the studies at a final concentration of 10 µM.

Table 4. Compounds used in the studies.

Compound	Name	Structure	CAS no.
Schisandrin lignans	Schisandrin		7432-28-2
	Schisandrin B		61281-37-6
	Schisandrin C		61301-33-5
MAPK inhibitors	SP600125		129-56-6
	SB203580		152121-47-6
	FR180204		865362-74-9

MAPK, Mitogen-activated protein kinase

4.2 Host cell lines

Human epithelial cell line, HL, obtained from Professor Pekka Saikku (National Institute of Health and University of Oulu, Finland) was originally isolated from the human respiratory tract [225]. HL cells were grown in Roswell Park Memorial Institute medium 1640 (RPMI 1640, (BioWhittaker/Lonza, Basel, Switzerland), supplemented with 7.5%

fetal bovine serum (FBS) (Lonza), 2 mM L-glutamine (Lonza) and 20 µg/ml gentamicin (Sigma-Aldrich).

Human monocytic cell line, THP-1 (ATCC TIB-202), originally isolated from acute monocytic leukemia patient, was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 Dutch modified medium (Gibco/Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Lonza), 2 mM L-glutamine (Lonza), 20 µg/ml gentamicin (Sigma-Aldrich) and 0.05 mM of mercaptoethanol (Gibco, NY, USA). When appropriate, THP-1 cells were differentiated into macrophages by incubating them for 48–72 h with 160 nM phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich).

Murine macrophage cell line, RAW264.7 (ATCC TIB-71), obtained from ATCC, originally isolated from an Abelson murine leukemia virus-induced tumor, were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco), supplemented with 10% FBS (Lonza) and 20 µg/ml gentamicin (Sigma-Aldrich).

All cell lines were cultured at 37 °C, 5% CO_2 and 95% humidity and stocks were stored in liquid nitrogen before use.

4.3 Chlamydial strains

Two strains of *C. pneumoniae* were used in the studies. The clinical isolate Kajaani 7 (K7), was originally isolated from the respiratory tract of a male patient during a *C. pneumoniae* epidemic in military facilities in Finland [226] (I). The other clinical isolate CV6, was originally isolated from a vasculature of a patient with coronary artery disease [227] (II, III, IV).

4.4. Propagation of *C. pneumoniae*

C. pneumoniae strains CV6 and K7 were propagated in HL cells. The cells were inoculated with *C. pneumoniae* in an appropriate amount of propagation medium (RPMI 1640 supplemented with 7.5% FBS, 2 mM L-glutamine, 0.6 µg/ml cycloheximide (Sigma-Aldrich), 10 µg/ml gentamicin, 100 mg/ml streptomycin (Sigma-Aldrich) and 3.75 µg/ml amphotericin B (Sigma-Aldrich) and incubated for 2.5 h at 37 °C. The medium was then supplemented to a volume appropriate for the vessel and the cells were incubated for additional 70 h. Finally the cells were lysed, cell debris was removed (10 min, 500 *g,* 4 °C), EBs were collected by pelleting (60 min, 21 000 *g*, 4 °C) and resuspended into sucrose-phosphate-glutamic acid (SPG) and stored at -80 °C. The EB stock titers were determined by infecting the HL cells (see section 4.5) cultured on coverslips, with 10-fold serial dilutions. Following a 72-h incubation, *C. pneumoniae* inclusions were stained with a genus-specific anti-LPS antibody (Pathfider, Bio-Rad, Hercules, CA, USA) and counted using the EVOS Cell Imaging System (Thermo Fisher Scientific).

4.5 Infections (I, II, III, IV)

Cells were seeded into 96-, 24- or 6-well plates at a density specific for each experiment (Table 5). The cells, grown as monolayers, were allowed to adhere to the surface overnight before infection, while suspension cells were infected immediately after seeding. The bacterial inoculum, with variable multiplicities of infection (MOI) (Table 6), was added to the wells in a small volume (e.g. 200 µl into 24-well) (Table 5) and the plates were centrifuged at 550 *g*, 60 min, at room temperature. The cells were then further incubated for 1 h, at 37 °C, after which the inoculum was removed and the test compounds added to the wells.

Table 5. Cell amounts and *C. pneumoniae* (Cpn) inoculums used in the studies.

Cell type	Well plate	Cell amount per well / liquid amount (µl)	Cpn inoculum (µl)
HL cells	6	-	-
	24	3×10^5 / 1000	200
	96	-	-
THP-1 monocytes	6	-	-
	24	$3–4 \times 10^5$ / 1000	1000
	48	5×10^5 / 1000	1000
	96	6×10^4 / 200	200
THP-1 macrophages	6	1×10^6 / 2000	1000
	24	4×10^5 / 1000	200
	96	6×10^4 / 200	100
RAW264.7	6	4×10^5 / 2000	-
	24	1.5×10^5	200
	96	-	-

Table 6. Multiplicities of infections (MOI) used in the experiments in this work.

Experiment / chapter	Cell line	MOI
Cytokine measurement / 4.7	THP-1 mono	2
NO measurement / 4.8	RAW 264.7	2
ROS measurement / 4.9	THP-1 macro	1; 5
GSH measurement / 4.10	THP-1 macro	5
Foam cell formation / 4.11	THP-1 macro	1
	RAW 264.7	1
Cholesterol measurement / 4.12	RAW 264.7	5
Gene expression analysis / 4.14	RAW 264.7	5
Coculture / 4.15	HL	1
EB production / 4.16.1	HL	1
EB exit / 4.16.1	HL	1
Infectivity / 4.16.2	THP-1 mono	1
Internalization / 4.16.3	THP-1 mono	1

4.6 Host cell viability assay (I, II, III, IV)

Host cell viability during treatment with the test compounds was determined by a resazurin viability assay. Cells were seeded into a 96- or 24- well plate and incubated with the studied compounds for the specific exposure times of each experiment. Resazurin (20 µM) (Sigma-Aldrich) was added to the wells and after a 2-h incubation at 37 °C fluorescence was recorded at 570/590 nm with the Varioskan LUX plate reader (Thermo Fisher Scientific). Cell viability was calculated comparing the fluorescence values with the DMSO control.

4.7 Enzyme-linked immunosorbent assay (ELISA) (I)

THP-1 cells were seeded into 48-well plates, infected with *C. pneumoniae* K7 or exposed to the LPS of *E. coli* and treated with the studied compounds. DuoSet ELISA Development Systems kits (R&D Systems, Minneapolis, MN, USA) were used to detect target protein concentrations. Secreted cytokines were detected from cell supernatants. In general, 96-well plates were first coated with the appropriate capture antibody overnight at room temperature. Wells were then washed with buffer containing 0.05% Tween® 20 (Sigma-Aldrich) in PBS, blocked by reagent diluent consisting of 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS (1 h), and the samples and standards were applied. After a 2-h incubation the biotin-conjugated detection antibody was added, and the wells were further incubated for 2 h. The wells were washed and streptavidin-conjugated horse radish peroxidase (HPR) was added to the wells for 20 min, to bind the detection antibody. Finally, substrate solution was added, and the color formed in the reaction catalyzed by HRP was detected with the Varioskan LUX plate reader (Thermo Fisher Scientific) at 450 nm.

4.8 Nitrate quantification assay (I)

Nitric oxide (NO) has an extremely short half-life in cells [228], so the quantification of nitrate, a metabolite of NO, is conducted instead. Cells were seeded into 24-well plates, infected with *C. pneumoniae*, K7 or CV6, and incubated with the studied compounds. Nitrate was quantified using Griess reagent, i.e. 1:1 of 0.1% *N*-1-napthylethylenediamine dihydrochloride (Sigma-Aldrich) and 2% sulfanilamide (Sigma-Aldrich) in 5% phosphoric acid, which reacts with nitrite forming a violet product. To this end, 100 µl of cell medium and 100 µl of Griess reagent were mixed in 96-well plate and the absorbance was recorded at 540 nm with Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific).

4.9 Intracellular ROS detection assay (I, II)

To determine the intracellular ROS levels, THP-1 were seeded into 96-well plate and exposed to the studied compounds with or without *C. pneumoniae* CV6 infection. Cells were loaded with dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich), which is taken into the cells and hydrolyzed into DCFH. In the presence of ROS, DCFH is oxidized to the fluorescent product dichlorofluorescein (DCF) which was detected with Varioskan LUX plate reader (Thermo Fisher Scientific) at 503/523 nm.

4.10 Glutathione quantification (I, II, IV)

The intracellular GSH levels of the cells were determined with the enzymatic recycling method, described by Rahman et al. [229]. The method is based on the reaction between GSH and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), that produces glutathione-TNB (GS-TNB) and TNB chromophore, whose amount can be measured. GS-TNB is reduced by glutathione reductase (GR), in the presence of NADPH, and GSH is taken back into the reaction. The present GSSG (oxidized GSH) is also reduced by GR and thus the total

GSH can be determined. THP-1 cells, either monocytes or macrophages, or RAW264.7 cells were seeded into 6- or 24-well plates and exposed to the studied compounds and/or *C. pneumoniae* CV6, for different time periods. Cells were collected and lysed using Triton X-100 (Sigma-Aldrich) and sulfosalicylic acid (Sigma-Aldrich) buffer. GR (Sigma-Aldrich) and DTNB (Sigma-Aldrich) were simultaneously added to the reaction, and after 30 sec β-NADPH (Sigma-Aldrich) was added. The formed TNB chromophore was detected with the Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific) set to 412 nm. GSH concentrations of the samples were obtained by linear regression from an 8-point standard curve.

To normalize the data, total protein concentrations were determined by acetone precipitation. 100 µl of the cell lysate samples were heated for 5 min at 95 °C, after which 400 µl of ice-cold acetone (Sigma-Aldrich) was added to the sample, followed by a 1-h incubation at -20 °C. Proteins were collected by centrifugation at 15 000 *g* and diluted in 100 nM Tris-buffer (Sigma-Aldrich). Concentrations were detected with Multiskan Sky microplate spectrophotometer (Thermo Fisher Scientific) using the µDrop plate (Thermo Fisher Scientific).

4.11 Oil Red O staining (IV)

THP-1 macrophages or RAW264.7 cells were cultured on 24-well plates, infected with *C. pneumoniae* CV6, and incubated with the studied compounds in the presence of 25 µg/ml of LDL (Sigma-Aldrich) for 48 h. The cells were then washed with PBS and fixed with 4% paraformaldehyde (PFA in PBS) (Santa Cruz Biotechnology, Dallas, TX, USA). The fixed cells were washed with Milli-Q water and incubated 5 min with 60% of isopropanol (Rathburn Chemicals, Walkerburn, Scotland). The cells were allowed to dry, and Oil Red O solution containing 350 mg of Oil Red O (Sigma-Aldrich) and 100 ml of 100% isopropanol, diluted 3:2 with Milli-Q water was added to the cells for 10 min. The dye was removed and the cells were washed with Milli-Q water until the

washing solution was clear. The cells and lipid droplets were analyzed under the EVOS cell imaging systems microscope (Thermo Fisher Scientific) using 20× magnification.

4.12 Free cholesterol and cholesteryl ester quantification (IV)

RAW264.7 macrophages were seeded into 24-well plates, infected with *C. pneumoniae* CV6 and incubated with the studied compounds, in the presence of 25 µg/ml LDL (Sigma-Aldrich). The ratio of free cholesterol and cholesteryl ester was determined from the cells with Amplex© Red cholesterol assay kit (Invitrogen/Thermo Fisher Scientific) by a method modified from the manufacturer's instructions. In brief, the cells were fixed with 2% PFA and washed with PBS. Lipids were extracted by incubating the cells for 30 min at 4 °C with ethanol. Samples were prepared by mixing 40 µl of the ethanol-lipid solution and 60 µl of the supplied reaction buffer. For detecting the amount of total cholesterol, 50 µl of samples were mixed with 50 µl of assay solution (150 µM Amplex© Red solution, 1 U/ml HRP, 1 U/ml cholesterol oxidase, 0.1 µM cholesterol esterase and reaction buffer). For the quantification of free cholesterol, the sample was incubated with a similar assay solution but without cholesterol esterase. Both solutions were incubated 30 min at 37 °C and the fluorescence was recorded with Varioskan Lux plate reader (Thermo Fisher Scientific) at 540/590 nm. The cholesterol concentrations were determined from a six-point standard curve. The fraction of esterified cholesterol was calculated based on the concentrations of the free and the total cholesterol.

4.13 Quantitative PCR (II, III)

Genomic DNA of the cells was extracted with GeneJet Genomic DNA purification kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The DNA concentrations were measured using NanoDrop (Thermo Fisher Scientific) or Multiskan Sky (Thermo Fisher Scientific) using the µDrop plate (Thermo Fisher Scientific) and 20 ng of DNA was added to each PCR reaction. The *C. pneumoniae* CV6 genome copy

numbers were quantified based on the *ompA* gene [230] using Step One Plus Real-Time PCR system (Thermo Fisher Scientific). Experiments were carried out using Fast SYBR Green master mix (Thermo Fisher Scientific) and 20 µl reactions were performed in 96-well MicroAmp optical plates (Thermo Fisher Scientific). Conditions in the PCR thermal cycler were 95 °C for 20 s and 40 cycles of 95 °C for 3 s and 60 °C for 30 s. *C. pneumoniae* genome equivalent (GE) quantity was determined comparing the results to a 5-point standard curve made from a chlamydial stock solution with known titer.

4.14 Reverse transcription PCR (IV)

RAW264.7 cells were seeded into 24-well plates and treated with the studied compounds, with or without *C. pneumoniae* CV6 infection. Total RNA of the cell culture samples was purified using PureLink RNA Mini Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. RNA was then used as a template for cDNA synthesis, which was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA concentrations were measured with NanoDrop (Thermo Fisher Scientific) and 20 ng were used per PCR reaction. The expression of different genes was quantified with the Step One Plus Real-Time PCR system (Thermo Fisher Scientific) and values were normalized to expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) (Thermo Fisher Scientific). The results for comparative ΔCt RT-PCR were obtained using the 2^-ΔΔCt method. In the data analysis, fold changes (RQ values) below 0.5 and above 2 were considered as significant. All primers used in the studies are listed in Table 7.

Table 7. Primers used in the studies.

Gene	genomic/ cDNA	Organism	Primer
ompA	genomic	*C. pneumoniae*	F: 5'- TCC GCA TTG CTC AGC C -3' R: 5'- AAA CAA TTT GCA TGA AGT CTG AGA A -3'
CD36	cDNA	mouse	F: 5'- TCG GAA CTG TGG GCT CAT TG -3' R: 5'- CCT CGG GGT CCT GAG TTA TAT TTT C -3'
PPARγ	cDNA	mouse	F: 5'- GAC ATC CAA GAC AAC CTG CTG -3' R: 5'- GCA ATC AAT AGA AGG AAC ACG -3'
GCLc	cDNA	mouse	F: 5'- ATG TGG ACA CCC GAT GCA GTA TT -3' R: 5'- TGT CTT GCT TGT AGT CAG GAT GGT TT -3'
GCLm	cDNA	mouse	F: 5'- GCC ACC AGA TTT GAC TGC CTT T -3' R: 5'- CAG GGA TGC TTT CTT GAA GAG CTT -3'
ABCA1	cDNA	mouse	F: 5'- CAA CTA CAA AGC CCT CTT TG -3' R: 5'- CTT GGC TGT TCT CCA TGA AG -3'
GGT-1	cDNA	mouse	F: 5'- GGA GAG AGT TTC TGC CCA TCC ATA C -3' R: 5'- GCG GCT GGG TGG GTG GT -3'

F, forward; R, reverse. All the primers used against mouse cDNA were checked with Primer-blast (NCBI) to confirm the absence of cross-recognition in *C. pneumoniae*.

4.15 Coculture assay (III)

HL cells were seeded into a 24-well plate (3×10^5 cells/well) and infected with *C. pneumoniae* CV6. At 67 h post infection the HL cells were washed with PBS and the same number of THP-1 monocytes were added. Studied compounds were added to the wells and the cells were incubated together for 5 h. THP-1 monocytes were pipetted from the wells, centrifuged and resuspended in 100 µl buffer solution containing 1% BSA and 5 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) in PBS. A solution of 1:100-diluted biotin-conjugated human leukocyte antigen (HLA-A2) antibody (Biolegend, CA, USA) was added and the cells were incubated on ice for 15 min,

followed by a washing with the buffer. The cells were then incubated on ice with a solution of 1:100-diluted streptavidin-conjugated MojoSort magnetic nanobeads (Biolegend). After that, the cells were washed and resuspended in 500 µl of the buffer. The cells were loaded into MidiMACS magnetic separator system (Miltenyi Biotech, CA, USA), washed with 4 x 3 ml of the buffer, eluted and counted. The summary of cell separation protocol is presented in Figure 4.

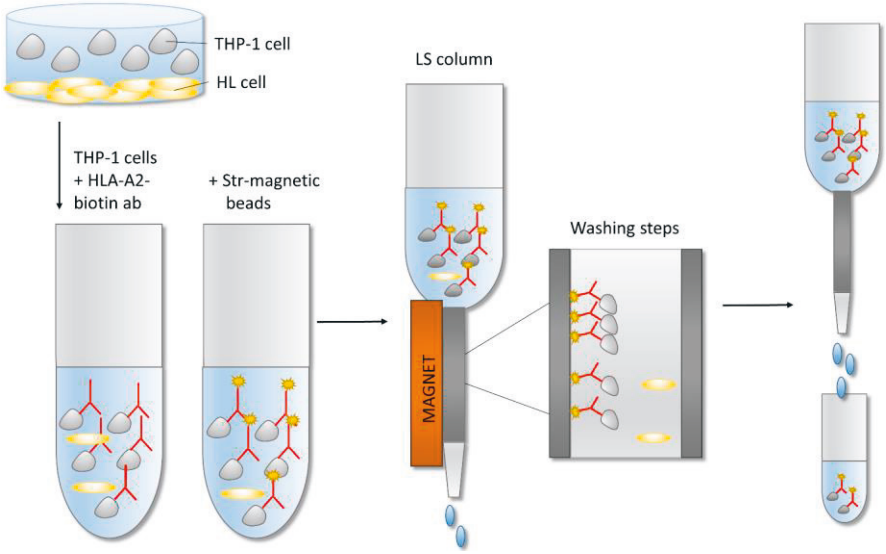


Figure 4. Coculture cell separation protocol using MACS magnetic bead separation system [231].

4.16 Infectivity assays (III)

4.16.1 Quantification of *C. pneumoniae* EB production and EB exit

HL cells were seeded into 24-well plates, infected with *C. pneumoniae* CV6, and incubated for 67 h at 37 °C. The studied compounds were added to the culture and the cells were incubated for additional 5 h at 37 °C. *C. pneumoniae* EBs released from the

cells were collected with the supernatant and the EBs remaining in the cells were harvested by scraping the cells and lysing them with glass beads. Both the EBs from the supernatants and from the cell lysates were inoculated onto fresh HL monolayer. After the EBs were allowed to internalize into the cells for 3 h, the cells were collected, and total cellular DNA was extracted with GeneJet Genomic DNA purification kit (Thermo Fisher Scientific). The *C. pneumoniae* genome copy numbers in the cells were quantified as described under the section 4.13.

4.16.2 *C. pneumoniae* EB infectivity assay

Purified *C. pneumoniae* CV6 EBs from a stock suspension were incubated with the studied compounds on ice for 2 h. THP-1 monocytes were added and the suspension was incubated for 2 h at 37 °C. The monocytes were collected in PBS and lysed, after which DNA was extracted with GeneJet Genomic DNA purification kit (Thermo Fisher Scientific). The *C. pneumoniae* genome copy numbers in the cells were quantified as described under the section 4.13.

4.16.3 *C. pneumoniae* EB internalization assay

A suspension of THP-1 cells, *C. pneumoniae* CV6 EBs and the studied compounds was incubated together for 2 h at 37 °C. The cells were washed and collected in PBS and DNA was extracted with GeneJet Genomic DNA purification kit (Thermo Fisher Scientific). The *C. pneumoniae* genome copy numbers internalized into the cells were quantified as described under the section 4.13.

4.17 Statistical analyses

Data were analyzed using the IBM SPSS Statistics v. 25 software (IBM, Armonk, NY, USA). The comparisons of group means were performed either by using Student's *t*-test, with Bonferroni correction where appropriate, or one-way ANOVA with Dunnett's T and Games-Howell post hoc tests for equal and unequal variance, respectively. *P*-values below 0.05 were considered as statistically significant and are indicated in the figures as *. *P*-values below 0.01 are indicated with **, and values below 0.001 are indicated with ***.

5. Results

5.1 The impact of schisandrin lignans on host cell viability (I, II, III, IV)

The impact of schisandrin lignans on host cell viability was studied with a resazurin viability assay. The viability of THP-1 monocytes, THP-1 macrophages, and RAW264.7 cells is presented as viability percentage in Table 8. No reduction in THP-1 monocyte cell viability was observed after 24 or 48-h exposure to any of the compounds or concentrations. The maximum incubation time in experiments involving THP-1 monocytes is 48 h. Neither schisandrin nor schisandrin C reduced the THP-1 macrophage cell viabilities, but schisandrin B caused a statistically significant reduction. This should be taken into consideration when evaluating the results concerning schisandrin B in THP-1 macrophages. Schisandrin B at a 50 µM concentration reduced also the RAW264.7 cell viability significantly after a 72-h incubation. Thus, most of the experiments with RAW264.7 cells were performed with a maximum of 48-h incubation. In the experiments with 72-h incubation, the possible host cell toxicity should be considered. The viability of HL cells after exposure to schisandrin lignans has been determined previously [203] and none of the studied lignans reduced the viability.

Table 8. The impact of schisandrin lignans on host cell viability.

Cell line	Compound	Viability-%		
		24 h	48 h	72 h
THP-1 monocytes	Schisandrin 25 µM	103 ± 15	145 ± 5	nd
	Schisandrin 50 µM	110 ± 7	127 ± 3	nd
	Schisandrin B 25 µM	163 ± 9	152 ±7	nd
	Schisandrin B 50 µM	103 ± 15	136 ± 5	nd
	Schisandrin C 25 µM	130 ± 3	128 ± 5	nd
	Schisandrin C 50 µM	70 ± 4	126 ± 5	nd
THP-1 macrophages	Schisandrin 25 µM	118 ± 8	105 ± 7	93 ± 2
	Schisandrin 50 µM	106 ± 7	119 ± 7	92 ± 6
	Schisandrin B 25 µM	80 ± 5	70 ± 8	80 ± 4*
	Schisandrin B 50 µM	67 ± 3*	50 ± 5*	69 ± 8
	Schisandrin C 25 µM	89 ± 7	78 ± 7	111 ± 6
	Schisandrin C 50 µM	79 ± 4	69 ± 4	101 ± 8
RAW264.7	Schisandrin 25 µM	nd	98 ± 1	100 ± 3
	Schisandrin 50 µM	nd	99 ± 2	95 ± 2
	Schisandrin B 25 µM	nd	87 ± 1	80 ± 4
	Schisandrin B 50 µM	nd	61 ± 3	36 ± 3*
	Schisandrin C 25 µM	nd	103 ± 3	97 ± 1
	Schisandrin C 50 µM	nd	101 ± 2	90 ± 1

nd, not determined. Viability studies were carried out with resazurin assay and the viability percentage are presented as mean ± SEM. Statistical significances were determined by Student's *t*-test with Bonferroni correction and they are presented as marks of *P* values: < 0.05: *; < 0.01: **; < 0.001: ***, n ≥ 3. Data from THP-1 monocytes are originally presented in publication I, THP-1 macrophages in publication II and RAW264.7 in publication IV.

5.2 The impact of schisandrin lignans on inflammatory cytokine production in THP-1 monocytes (I)

The impact of schisandrin lignans on inflammatory cytokine production was determined in THP-1 monocytes infected with *C. pneumoniae* K7 or challenged with *E. coli* LPS using ELISA. The concentrations of three interleukins, IL-6, IL-10 and IL-12 were detected. As a response to *C. pneumoniae* infection, only the levels of IL-12 were elevated in THP-1 monocytes (Figure 5). The LPS of *E. coli*, which is considered more

immunogenic than *C. pneumoniae* LPS, elevated both IL-12 and IL-6 levels. The IL-12 elevation was approximately 20-fold higher in the LPS-stimulated samples than in *C. pneumoniae* samples. Neither exposure elevated the IL-10 levels in THP-1 monocytes.

Schisandrin had no effect on the levels of any of the studied interleukins. Schisandrin B, on the other hand, decreased the secretion of all the studied interleukins compared to the LPS or *C. pneumoniae* controls (CPN). At 25 µM, it reduced the IL-12 levels by 40% (LPS) and 69% (CPN). The IL-6 levels were reduced by 44% (LPS). At 50 µM concentration the reduction in IL-12 levels was 89% (LPS) and 68% (CPN), while the reduction in IL-6 levels was 49% (LPS). Schisandrin C also decreased the LPS induced IL-12 production at 50 µM concentration by 68%, and *C. pneumoniae*-induced IL-12 at 25 µM by 65%. IL-6 (LPS) was reduced by 32% at 25 µM concentration and 35% at 50 µM concentration.

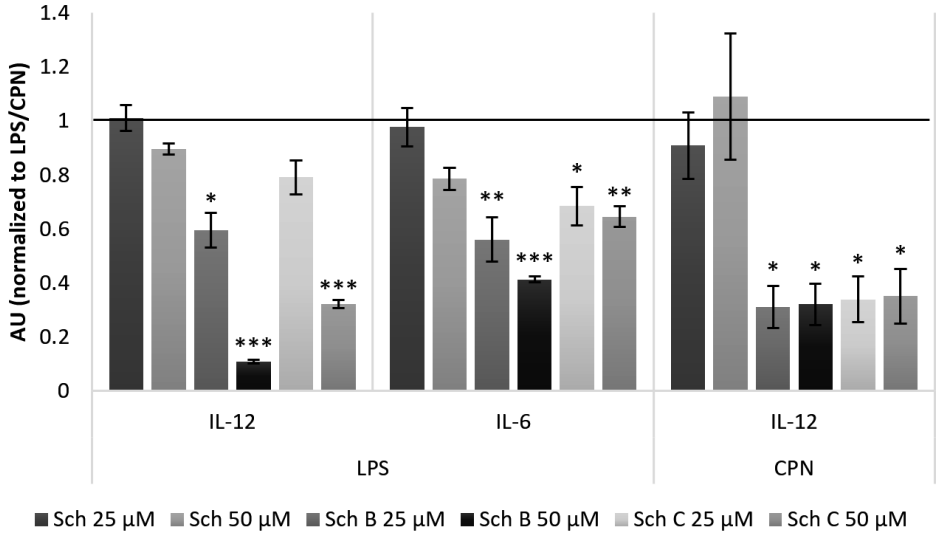


Figure 5. IL-12 and IL-6 production in THP-1 monocytes after LPS and *C. pneumoniae* exposure and treatment with schisandrin, schisandrin B and schisandrin C. Data are presented as secretion index, compared to LPS or *C. pneumoniae* infection control, as mean ± SEM. Statistical significances were determined using ANOVA and Games-Howell post hoc test (p : < 0.05: *; < 0.01: **; < 0.001: ***), n ≥ 4. Data are originally presented in publication I.

5.3 The impact of schisandrin lignans on NO production of the cells (I)

The nitrate levels, indicative of NO, were quantified in THP-1 monocytes using the Griess reagent. The cells were treated with 1 µg/ml of LPS of *E. coli* and the nitrate levels were determined spectrophotometrically.

There was no elevation of nitrate levels observed in THP-1 cells, neither monocytes nor macrophages, even after exposure to high levels of LPS or *C. pneumoniae* CV6 infection at any time point between 4 h and 72 h (data not shown). Some controversial data exist regarding the topic, but there are studies that are in line with our results [232, 233] that NO is indeed not produced in THP-1 cells.

Further nitrate production studies were thus conducted with RAW264.7 macrophage cells. The cells were treated with 1 µg/ml of LPS and they were incubated with 50–100 µM of schisandrin, schisandrin B and schisandrin C, with luteolin (10 µM) as the positive control. The results shown in Figure 6A demonstrate that schisandrin B and schisandrin C inhibited the LPS-induced nitrate production after a 24-h exposure by 86% and 42%, respectively.

The impact of schisandrin lignans (25 µM and 50 µM) was also studied in RAW264.7 macrophages infected with *C. pneumoniae* and compared to the LPS-induced nitrate production. The cells were incubated with schisandrin lignans for 24, 48 and 72 h. *C. pneumoniae* infection elevated the nitrate levels significantly compared to the untreated control (Figure 6B). Schisandrin had no effect on *C. pneumoniae*-induced nitrate levels, but schisandrin B reduced them at both concentrations. At 24, 48 and 72 h, 25 µM schisandrin B had reduced the levels by 45%, 50% and 50%, respectively. Treatment at a higher 50 µM concentration reduced the levels by 61%, 74% and 76%, respectively.

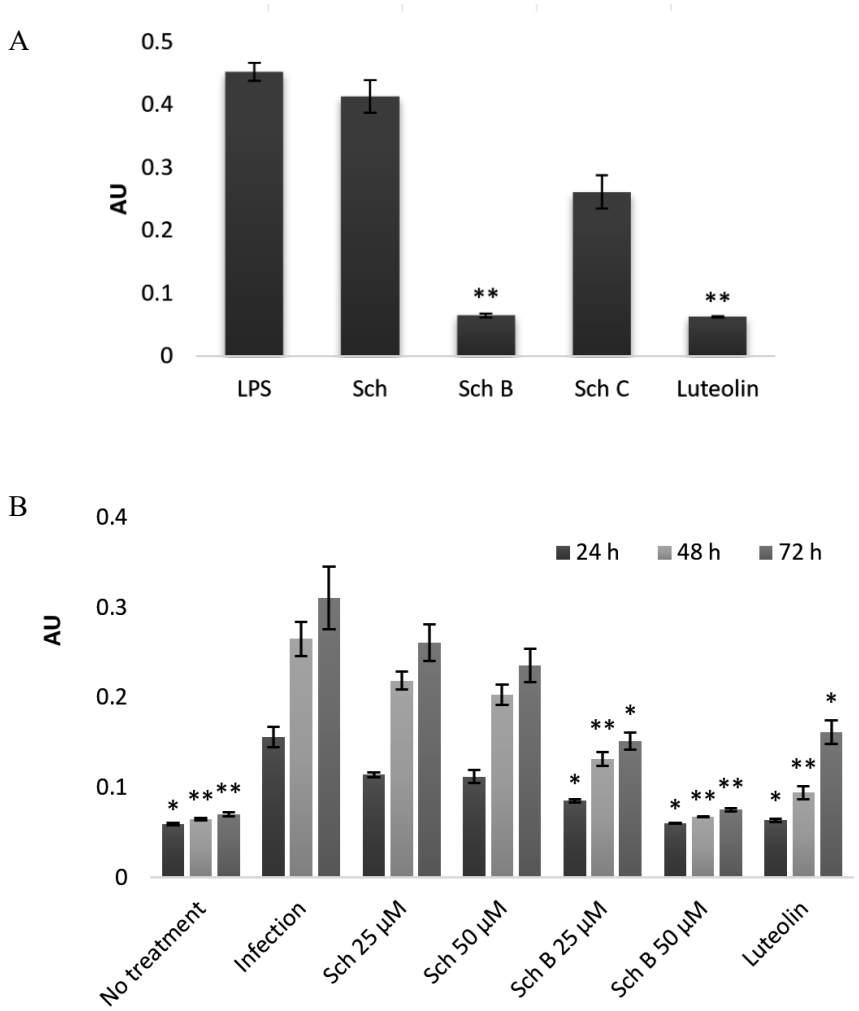


Figure 6. Nitrate production in RAW264.7 cells after A) LPS exposure and B) *C. pneumoniae* infection. The cells were treated with schisandrin lignans and luteolin after the induction of nitrate production. Data are presented as mean ± SEM. Statistical significances were determined using ANOVA and Games-Howell post hoc test ($p < 0.05$: *; < 0.01: **; < 0.001: ***), $n \geq 3$. Unpublished data.

5.4 ROS production in THP-1 cells (I, II)

5.4.1 The impact of *C. pneumoniae* infection and LPS exposure on ROS levels

The impact of *C. pneumoniae* CV6 infection on the intracellular ROS production of THP-1 macrophages was studied. The cells were infected at MOI1 and MOI5 and treated with 1 µg/ml of LPS of *E. coli*. After 24, 48 or 72 h incubation the cells were exposed to DCFH-DA for 30 min and fluorescence was detected. Results were compared to non-infected control. *C. pneumoniae* elevated the ROS levels after 48 and 72 h and the elevation was MOI-dependent (Figure 7). After 48 h the increase was 18% (MOI1) and 26% (MOI5), and after 72 h it was 25% (MOI1) and 34% (MOI5). LPS of *E. coli* also elevated the ROS levels after 48 h (75%) and 72 h (47%).

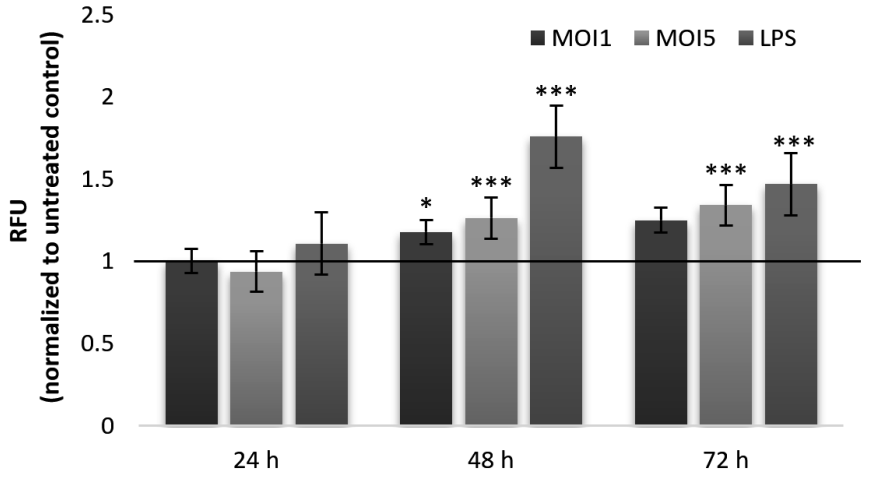


Figure 7. ROS levels in THP-1 macrophages after 24, 48 and 72 h exposure to 1 µg/ml of LPS of *E. coli*, MOI1 or MOI5 *C. pneumoniae* infection. Data are presented as mean ± SEM. Statistical significance was determined using Student's *t*-test with Bonferroni correction ($p < 0.05$: *; < 0.01: **; < 0.001: ***), $n \geq 4$. Data are originally presented in publication II.

5.4.2 The impact of schisandrin lignans on basal ROS levels

The impact of schisandrin lignans on the basal ROS production of THP-1 monocytes and macrophages was additionally studied. In monocytic cells, the immediate ROS production after lignan exposure was studied. The cells were loaded with DCFH-DA, after which they were treated for 1 h. As shown in Figure 8A, schisandrin at both 25 and 50 µM concentrations elevated the ROS levels significantly; 25 µM by 43% and 50 µM by 50%. There were no differences in the quantified ROS levels after schisandrin B and schisandrin C exposure compared to the vehicle control.

In THP-1 macrophages, the impact of schisandrin lignans on intracellular ROS production was determined at 4 to 72 h. There were no differences after 4–48 h of lignan treatment, but at 72 h schisandrin and schisandrin C had significantly elevated the ROS levels (Figure 8B). Schisandrin elevated the levels by 31% at both concentrations and schisandrin C elevated them by 77% (25 µM) and 91% (50 µM).

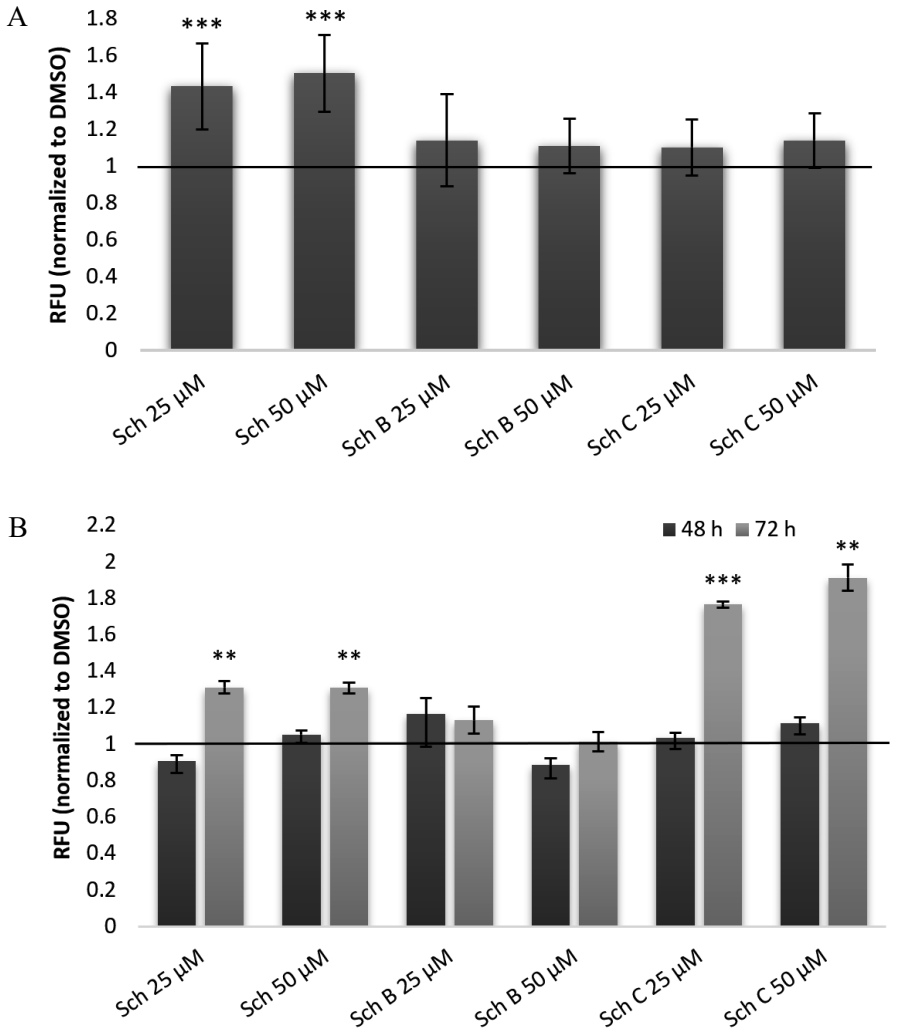


Figure 8. Impact of schisandrin lignans on ROS levels in THP-1 cells: A) 30 min exposure with THP-1 monocytes and B) 48 and 72-h incubation with THP-1 macrophages. Data are presented as mean ± SEM. Statistical significance was determined using Student's *t*-test with Bonferroni correction ($p < 0.05$: *; < 0.01: **; < 0.001: ***), $n \geq 4$. Data from THP-1 monocytes is originally presented in publication I and from THP-1 macrophages in publication II.

5.4.3 The impact of schisandrin lignans on ROS levels in *C. pneumoniae*-infected and LPS-treated THP-1 macrophages

The impact of schisandrin lignans on ROS levels was determined in THP-1 macrophages after LPS (*E. coli*) exposure (Figure 9A) and *C. pneumoniae* CV6 infection (Figure 9B). In LPS-treated cells, schisandrin B decreased the levels significantly at both concentrations, by 17% (25 µM) and 56% (50 µM), and schisandrin C decreased the levels at 50 µM by 32%. Schisandrin had no effect on the LPS-induced ROS levels. On the other hand, only 50 µM schisandrin B decreased the *C. pneumoniae*-induced ROS production by 43% (48 h) and 64% (72 h). THP-1 macrophage viability was significantly decreased with 50 µM schisandrin B (Table 8), which may have an influence on these results.

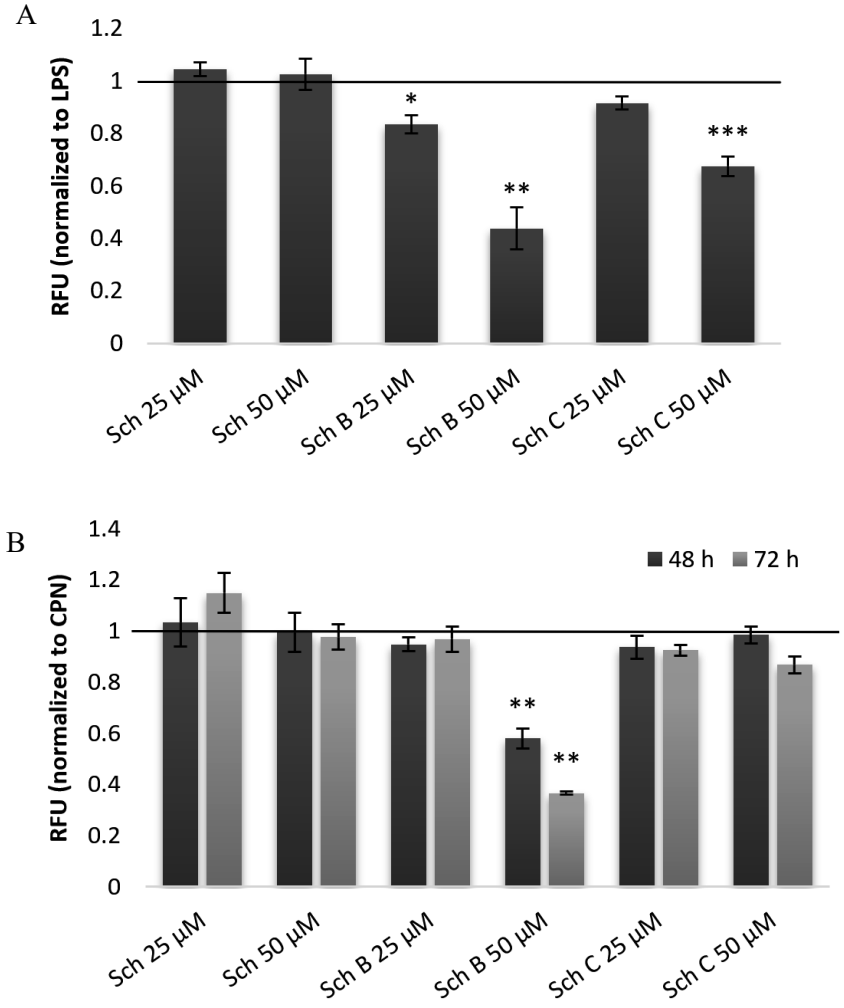


Figure 9. The impact of schisandrin lignans on A) LPS-induced and B) *C. pneumoniae*-induced ROS production. Data are presented as mean ± SEM. Statistical significance was determined using Student's *t*-test with Bonferroni correction ($p < 0.05$: *; < 0.01: **; < 0.001: ***), $n \geq 4$. Data from LPS-induced ROS production are unpublished and data from *C. pneumoniae*-induced ROS production are originally presented in publication II.

5.5 The impact of schisandrin lignans on cellular GSH levels (I, II, IV)

5.5.1 The impact on basal GSH levels

The effect of schisandrin lignans on intracellular GSH was determined in THP-1 cells, both monocytes and macrophages, and in RAW264.7 macrophages. All the results were compared to the vehicle control and the levels in both macrophages were normalized to the total protein amount of the samples.

The cells were cultured in 6-well plates and exposed to schisandrin lignans for different time periods. Total GSH levels in the cells were detected using enzymatic recycling method with DTNB. In THP-1 monocytes, schisandrin B decreased the levels after 24 h by 49% (50 µM) and after 48 h by 54% (25 µM) and 73% (50 µM) (Figure 10). Schisandrin C (50 µM) also decreased the levels by 50% after 48 h. Furthermore, schisandrin B and C had significantly decreased the GSH-levels already after 4 h incubation (data not shown). Schisandrin had no significant effect on GSH levels in THP-1 monocytes.

In THP-1 macrophages, schisandrin B decreased the levels after 24 h by 31% (25 µM) and after 48 h by 76% (25 µM) and 91% (50 µM). Schisandrin (50 µM) reached a significant decrease (47%) by the 24 h time point. Schisandrin C also seemed to decrease the GSH levels in THP-1 macrophages, but the effect was not statistically significant.

In RAW264.7 macrophages, the impact of schisandrin lignans was different compared to THP-1 cells. Schisandrin at 50 µM decreased the GSH levels 54% and 73% after 48 h and 72 h, respectively. Schisandrin C elevated the levels after 48 h by 76% (25 µM) and 110% (50 µM). The higher concentration elevated the levels significantly also after 72 h incubation (64%). While schisandrin B seemed to elevate the levels at each time point as well, the change did not reach statistical significance.

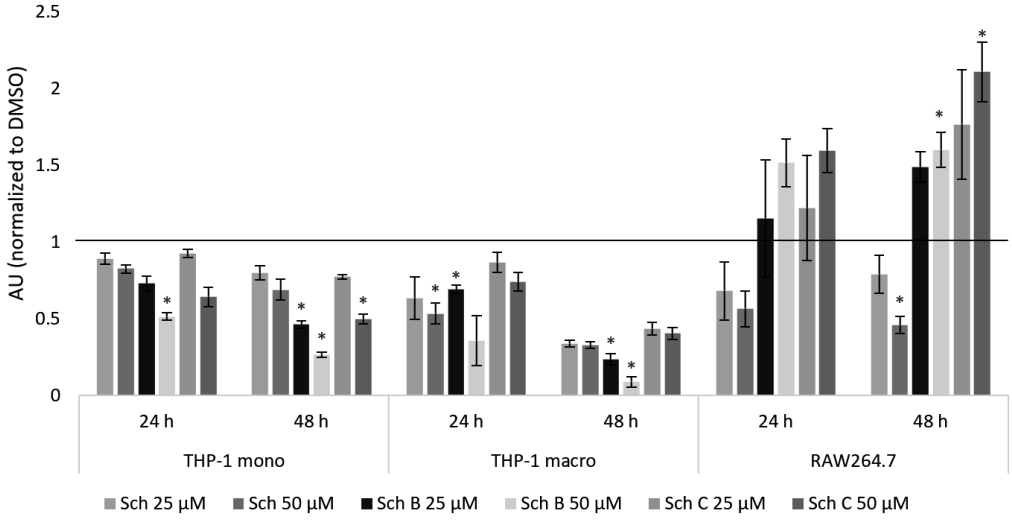


Figure 10. The impact of schisandrin lignans on basal GSH levels in THP-1 monocytes and macrophages and RAW264.7 macrophages. The cells were incubated with the compounds for 24 and 72 h and the GSH levels were compared to vehicle control (DMSO). Data are presented as mean ± SEM. Statistical significance was determined using Student's *t*-test with Bonferroni correction ($p < 0.05$: *; < 0.01: **; < 0.001: ***), $n \geq 4$. Data from THP-1 monocytes are originally presented in publication I, from THP-1 macrophages in publication II and from RAW264.7 in publication IV.

5.5.2 The impact of schisandrin lignans on the GSH levels of *C. pneumoniae*-infected THP-1 macrophages

The impact of *C. pneumoniae* CV6 infection on GSH levels in THP-1 macrophages and the effects of schisandrin, schisandrin B and schisandrin C on those levels was next determined. The cells were infected with MOI1 and MOI5 for 48 and 72 h. While MOI1 elevated the GSH levels significantly at 72 h incubation, MOI5 did so already at 48 h, but the effect was mitigated later. Schisandrin decreased the *C. pneumoniae*-induced GSH levels at 25 and 50 µM concentrations after a 72-h incubation by 62% and 57%, respectively (Figure 11). Schisandrin B decreased the levels at both concentrations after 48 (71% and 99%) and 72 h (69% and 84%), while schisandrin C decreased the levels at both concentrations after 72 h incubation by 65% and 55%, respectively.

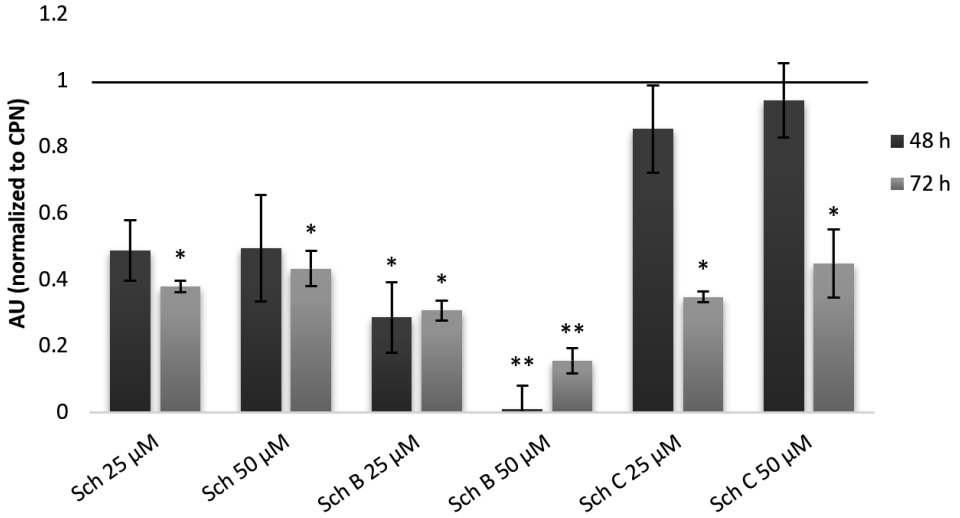


Figure 11. The impact of schisandrin lignans on the GSH levels of *C. pneumoniae*-infected THP-1 macrophages. The cells were incubated with the compounds for 48 and 72 h and the GSH levels are compared to vehicle control (DMSO). Data are presented as mean ± SEM. Statistical significance was determined using Student's *t*-test with Bonferroni correction ($p < 0.05$: *; < 0.01: **; < 0.001: ***), $n \geq 4$. Data are originally presented in publication II.

5.6 The impact of schisandrin lignans on *C. pneumoniae*-induced foam cell formation (IV)

RAW264.7 macrophages and THP-1 macrophages were infected with *C. pneumoniae* CV6 and treated with 50 µM schisandrin lignans in the presence of LDL for 48 h. The cells were stained with Oil Red O dye to examine the resulting foam cell formation. The effects of the glutathione biosynthesis inhibitor buthionine sulfoximine (BSO) and the MAPK inhibitors SP600125 (JNK1/2) and SB202190 (p38) on the foam cell formation was also studied. *C. pneumoniae* induced foam cell formation in both cell lines (Figure 12A and B). BSO increased the foam cell formation in RAW264.7 cells (Figure 12A), which is in line with earlier reports [234]. Schisandrin had no effect on lipid accumulation, but schisandrin B inhibited it very efficiently in both cell lines. Schisandrin C also blocked the lipid accumulation in RAW264.7 cells (Figure 12A), but

not completely in THP-1 cells (Figure 12B). SP600125, a JNK1/2 inhibitor, blocked the foam cell formation in RAW264.7 cells (Figure 12A) but not in THP-1 cells (Figure 12B). SB202190, a p38 kinase inhibitor, instead seemed to decrease lipid droplets in THP-1 cells, as compared to the untreated control.

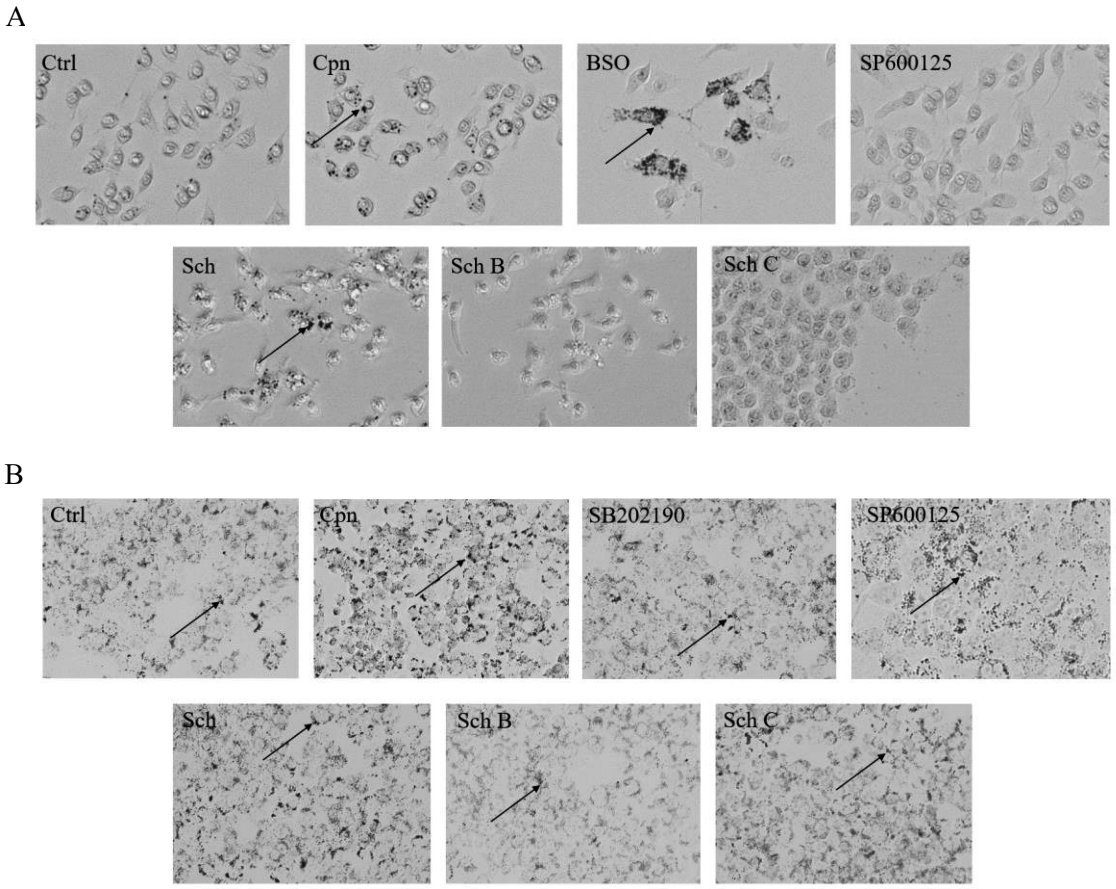


Figure 12. Impact of schisandrin lignans and MAPK inhibitors on macrophage foam cell formation in the presence of LDL in A) RAW264.7 cells and B) THP-1 cells. Lipid droplets in the cells are indicated with arrows. Data of RAW254.7 cells are presented in publication IV.

5.7 The impact of schisandrin lignans on total cholesterol and cholesteryl ester levels in RAW264.7 cells (IV)

The total cholesterol and cholesteryl ester levels were determined with Amplex© red reagent in infected RAW264.7 macrophages, treated with schisandrin lignans (50 µM) and BSO (250 µM) in the presence of 25 µg/ml of LDL, after a 48-h exposure. According to these data, BSO elevated the total cholesterol levels by 19.4% (p=0.004) while schisandrin B decreased the levels by 18.9% (p=0.005), as compared to the infection control (Figure 13). The cholesteryl ester levels were calculated by subtracting the free cholesterol level from that of the total cholesterol. Schisandrin B and schisandrin C seemed to lower the cholesteryl ester levels (21.9% and 19.7%, respectively), although these effects did not reach statistical significance.

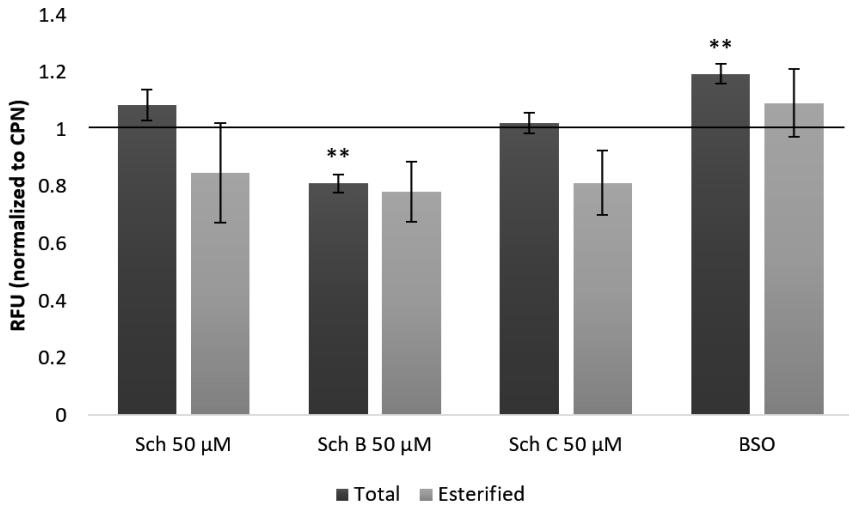


Figure 13. The impact of schisandrin lignans on total cholesterol and cholesteryl ester levels in RAW264.7 macrophages. The cells were incubated with schisandrin lignans for 48 h and cholesterol levels were compared to the infection control. Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Dunnett's T post hoc test ($p < 0.05$: *; < 0.01: **; < 0.001: ***), n = 6. Data are presented in publication IV.

5.8 The impact of schisandrin lignans on the gene expression profile in RAW264.7 macrophages (IV)

The impact of schisandrin lignans on the expression of an array of GSH and lipid metabolism-related genes in RAW264.7 macrophages was studied using reverse transcription PCR. The cells were incubated for 48 h in the presence of 50 μM schisandrin, schisandrin B and schisandrin C. The expression levels of *PPARγ*, *CD36*, *ABCA1*, *GCLc*, *GCLm* and *GGT-1* were detected and compared to *GAPHD* housekeeping gene expression. The impact of *C. pneumoniae* CV6 infection and the concomitant administration of schisandrin lignans was also studied by infecting the cells with a MOI5 and incubating them for 48 h.

PPARγ is a nuclear receptor which regulates lipid and glucose metabolism and its expression is changed in several pathological conditions, such as atherosclerosis. CD36 is a scavenger receptor that imports lipids, such as oxidized LDL and fatty acids, into the cells. ABCA1, on the other hand, mediates the efflux of cholesterol and phospholipids out of the cells. GCLc and GCLm are the two domains (catalytic and modifier) of the enzyme that is synthetizing GSH. GGT-1 is a protein that breaks up the extracellular GSH and it is a key regulator in the GSH synthesis.

The isolated effect of *C. pneumoniae* infection was first characterized to provide a baseline for the experimental treatment study. There were no changes in the gene expression after *C. pneumoniae* (MOI5) infection. However, when the infection was performed in presence of LDL, the *PPARγ* expression decreased statistically significantly, by RQ of 0.4 (Table 9). The expression levels of the other genes of interest remained unchanged.

The impact of schisandrin lignans on the gene expression of non-infected RAW264.7 cells is presented in Table 10. Schisandrin B and C elevated the expression of *PPARγ* by the relative quantification (RQ) of 4.7 and 2.3, respectively, while all three lignans had an effect on the *ABCA1* expression: schisandrin inhibited it (RQ 0.4) and schisandrin B and C increased it (RQ 15.2 and 4.0, respectively). Schisandrin B also increased the

expression of GSH metabolism-related genes *GCLc*, *GCLm* and *GGT-1* by RQ of 2.2, 2.6 and 4.5, respectively.

When the cells were concomitantly exposed to *C. pneumoniae* and the schisandrin lignans, the changes in gene expressions were almost similar to the samples only exposed to the lignans, with *ABCA1* being the most affected one (Table 10). Schisandrin downregulated *ABCA1* by a RQ of 0.3, whereas schisandrin B and schisandrin C caused a considerable upregulation, (RQ 10.1 and 5.3, respectively).

Table 9. The impact of *C. pneumoniae* infection on the gene expression profile in RAW264.7 cells.

Gene	Effect on expression	RQ value
PPARγ	decreasing	**0.4** ± 0.08*
CD36	-	0.9 ± 0.04
ABCA1	-	1.8 ± 0.4
GCLc	-	0.8 ± 0.06
GCLm	-	0.9 ± 0.09
GGT-1	-	0.6 ± 0.05

The amplification of each gene was compared to the housekeeping gene GAPHD. Relative quantification (RQ) values were obtained using the 2^-ΔΔCt method (Ct: cycle threshold). RQ values are presented (± SEM), and changes above 2 and below 0.5 were considered as significant and marked with bolding. Statistical significances were determined according to ΔCt with Student's *t*-test ($p < 0.05$: *; < 0.01: **; < 0.001: ***), n = 3. Data are presented in publication IV.

Table 10. The impact of schisandrin lignans on the expression of lipid and GSH metabolism genes in RAW264.7 cells, with and without *C. pneumoniae* infection.

Gene	Compound	RQ value / basal	RQ value / infection
PPARγ	Schisandrin	1.5 ± 0.5	1.2 ± 0.3
	Schisandrin B	**4.7** ± 0.8**	**6.5** ± 0.3**
	Schisandrin C	**2.3** ± 0.5	2.0 ± 0.5
CD36	Schisandrin	0.9 ± 0.2	0.8 ± 0.04
	Schisandrin B	1.6 ± 0.3	**2.2** ± 0.3
	Schisandrin C	1.7 ± 0.5	**2.0** ± 0.6
ABCA1	Schisandrin	**0.4** ± 0.1	**0.3** ± 0.05*
	Schisandrin B	**15.2** ± 1.3**	**10.1** ± 3.4**
	Schisandrin C	**4.0** ± 0.8	**5.3** ± 1.9*
GCLc	Schisandrin	1.2 ± 0.6	1.4 ± 0.05
	Schisandrin B	**2.2** ± 0.1*	**2.5** ± 0.3**
	Schisandrin C	1.5 ± 0.3	1.2 ± 0.2
GCLm	Schisandrin	1.5 ± 0.2	1.6 ± 0.2
	Schisandrin B	**2.6** ± 0.2**	**3.8** ± 0.4***
	Schisandrin C	1.9 ± 0.3*	1.8 ± 0.4*
GGT-1	Schisandrin	**2.1** ± 0.9	1.2 ± 0.2
	Schisandrin B	**4.5** ± 0.7*	**4.4** ± 1.2**
	Schisandrin C	1.2 ± 0.3	1.4 ± 0.4

The amplification of each gene was compared to the housekeeping gene GAPHD. Relative quantification (RQ) values were obtained using the 2^-ΔΔCt method (Ct: cycle threshold). RQ values are presented (± SEM) and changes above 2 and below 0.5 were considered as significant and marked with bolding. Statistical significances were determined according to ΔCt with ANOVA and Dunnett's T post hoc test ($p < 0.05$: *; < 0.01: **; < 0.001: ***), n = 3. Data are presented in publication IV.

5.9 The study of *C. pneumoniae* transfer from respiratory epithelium to monocytes (III)

C. pneumoniae is known to transfer from its primary site of an infection, respiratory epithelium, to other sites of the body, and by that mechanism to contribute to the development of chronic inflammatory diseases, such as atherosclerosis. A coculture model with HL cells and THP-1 monocytes was developed, in which the transfer of bacteria between the epithelial cells and phagocytes can be studied.

HL cell monolayers were infected with *C. pneumoniae* CV6. At the end of the infection cycle (67 h post infection), THP-1 cells and the studied compounds were added to the culture with the HL cells. The cells were incubated together for 5 h, after which THP-1 cells were selectively collected using the MACS magnetic cell separation system. Finally, the *C. pneumoniae* genome copy numbers were detected using qPCR.

The compounds used in the developing the system were the MAPK inhibitors SP600125 (JNK1/2 inhibitor), SB203580 (p38 inhibitor) and FR180204 (ERK inhibitor) whose targets are known to be affected by *C. pneumoniae* infection. SP600125 decreased the transfer of *C. pneumoniae* between the cell lines by 69%, SB203580 by 54% and FR180204 by 37%, as compared to the untreated infection control (Figure 13).

The possible mechanisms by which the MAPK inhibitors block the transfer of *C. pneumoniae* were examined. The results of the different infectivity studies are presented in Figure 14. The MAPK inhibitors did not have any effect on *C. pneumoniae* EB production inside the epithelial cells, but SP600125 significantly increased the release of the EBs from the cells. In addition, EBs were pre-treated with the MAPK inhibitors, whose influence on the EB infectivity was then examined. No effect on infectivity was observed. When THP-1 cells were incubated with MAPK inhibitors and *C. pneumoniae* EBs for 2h, the compounds SP600125 and SB203580 decreased the *C. pneumoniae* internalization by 40% and 38%, respectively. FR180204 also decreased the internalization by 28% but the decrease was not statistically significant.

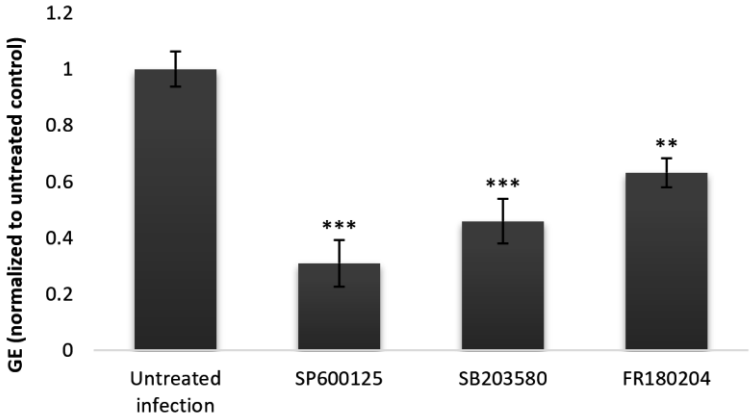


Figure 13. Impact of MAPK inhibitors on the transfer of *C. pneumoniae* between HL epithelial cells and THP-1 monocytes. *C. pneumoniae* genome copy numbers (GE) inside THP-1 monocytes were determined by qPCR. Data are presented as mean ± SEM. Statistical significance was determined using ANOVA and Dunnett's T post hoc test ($p < 0.05$: *; < 0.01: **; < 0.001: ***), n ≥4. Data are originally presented in publication III.

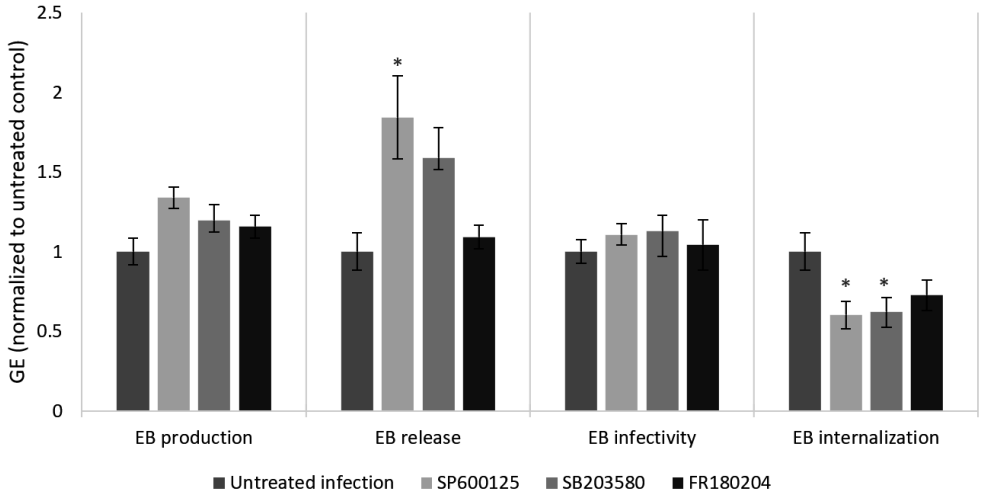


Figure 14. Impact of MAPK inhibitors on the production, release, infectivity and internalization of chlamydial elementary bodies (EB). *C. pneumoniae* genome copy numbers (GE) were determined by qPCR. Data are presented as mean ± SEM. Statistical significance was determined using ANOVA and Dunnett's T post hoc test ($p < 0.05$: *; < 0.01: **; < 0.001: ***), n ≥4. Data are originally presented in publication III.

6. Discussion

This thesis focused on antichlamydial lead compound profiling in monocyte and macrophage cell models. The compounds of interest were three schisandrin lignans, schisandrin, schisandrin B and schisandrin C, which are dibenzocyclooctadiene lignans isolated from *Schisandra chinensis* berries. The studies involved the human lung cell line (HL); THP-1 monocytic cell line, which was also differentiated into macrophage like cells; and RAW264.7 murine macrophage cell line.

Since *Chlamydia pneumoniae* is an obligate intracellular bacterium, all biological studies concerning the bacterium need to be done by infecting mammalian cells. Most cell lines used in *Chlamydia*-related drug research and susceptibility screenings are epithelial cell lines; such as HeLa, Hep2 and HL cells; as well as endothelial cell lines, such as HUVECs. These are permissive cell lines in which *Chlamydia* can replicate effectively and maintain a productive infection.

In vivo, C. pneumoniae can disseminate from the primary site of an infection, namely the respiratory tract, to other body sites through PBMCs [3, 18]. Via this route, bacteria can infect various tissues in the body, and these infections have been related to many chronic inflammatory diseases [235].

From a therapeutic point of view, PBMCs are of high importance because the infection in these cells is in a persistent stage [8], and it is thus very difficult to treat with conventional antibiotics. Being able to eliminate the pathogen in these cells and to prevent the changes induced in PBMC behavior would be important for stopping the pathological processes related to inflammatory diseases.

The persistence of *C. pneumoniae* is a massive problem in the clinic. Persistent infections remain unnoticed due to their minor or even absent symptoms, but on a cellular level, persistent infections still affect infection sites by maintaining the inflammatory processes in their surroundings [7]. Still, even if persistent *Chlamydia* were readily diagnosed,

there are no effective drugs against such form of infection, since all conventional antibiotics are ineffective against it.

In this work, I used the *C. pneumoniae* strains K7 and CV6 in persistent infection models to study their effects on monocytic and macrophage cell lines. No data were previously available on the impacts of schisandrin lignans on the persistent *C. pneumoniae*-induced functions in the cell lines used in this work. As another contribution to compound profiling studies I developed a new platform for studying the transfer of the infection between epithelial cells and phagocytes, which can be used in the preclinical drug studies of antichlamydial compounds.

Several antichlamydial compounds have been studied during the past decades, including already approved drugs such as statins [184], heparins [185] and rapamycin [236], as well as investigational compounds such as plant polyphenols [196], betulin derivatives [237], 2-arylbenzimidazoles [186] and $\beta^{2,2}$-amino acid derivatives [31]. Antibiotic treatments against *C. pneumoniae* and their impact on coronary artery disease and multiple sclerosis have also been studied previously.

Schisandra chinensis lignans have been studied in our laboratory for several years since they demonstrated antichlamydial activity in inhibition studies against *C. pneumoniae* and *C. trachomatis* in permissive cell lines. Compounds were chosen into these studies because they showed activity also in monocytes and macrophages where *C. pneumoniae* infection is persistent.

The *C. pneumoniae* cellular components in PBMCs are recognized by the host cells via the pattern-recognizing Toll like receptors (TLRs) and Nod-like receptors (NLRs) [133, 141]. The receptors are localized in cell membrane and cytoplasm, respectively, where they recognize various structures in microorganisms and initiate the immune responses. There are 10 members in TLR family identified in human cells (TLR1–10) and 12 in mice (TLR1–9, TLR11–13) [238]. Concerning *C. pneumoniae* recognition, TLR2 and TLR4 are the most relevant. It is reported that *C. pneumoniae* induces inflammatory molecule production through the TLR2 signaling cascade in 293HEK cells, dendritic

cells [85] and in macrophages [85, 132]. TLR4 has also been reported to initiate the *C. pneumoniae*-induced transactions in dendritic cells, human monocytes [239] and murine models [240]. TLR3 activation in the HCAEC primary cell line has also been suggested [241]. Most likely, various TLRs, at least TLR2, TLR3 and TLR4 are responsible for recognizing *C. pneumoniae* and initiating the proinflammatory signaling cascades [146]. The myeloid differentiation factor 88 (MyD88) is an adaptor protein that is involved in most TLR signaling cascades, and it has been reported to also take part in *C. pneumoniae*-induced TLR signaling [133, 141, 242, 243], while MyD88 independent signaling is also reported [133].

When TLRs get activated by the pathogen, they initiate the inflammation signaling cascade involving mitogen-activated protein kinases (MAPKs) [244] and it has been reported that *C. pneumoniae* also induces MAPK activation [245]. There are three main MAPKs that differentially regulate the inflammatory reactions: the extracellular signal-regulated protein kinase (ERK), the p38 MAP kinase (p38) and the c-Jun amino-terminal kinase (JNK) and they can be activated with different signaling molecules and stimuli, which are cell type-dependent [246, 247]. All three major MAPKs have been reported to be activated in *C. pneumoniae* infection [248]. On the other hand, Cheng et al. [149] reported that *C. pneumoniae* infection induced the phosphorylation of ERK, JNK1/2 and p38 MAPKs, but a p38 inhibitor did not inhibit the further signaling cascade.

In addition to pathogens, the activation of the MAPK signaling may occur as a response to reactive oxygen species (ROS) [249] that cause oxidative stress. Oxidative stress, i.e. the excess amount of oxidants, ROS and reactive nitrogen species (RNS) in the cells leads to various cellular dysfunctions and the initiation of inflammation, but also to the activation of the innate immune system [250]. Oxidative stress has also been related to *C. pneumoniae* infection in various ways [251]. On one hand, *C. pneumoniae* is reported to increase the levels of ROS and RNS in the cells, which is a mechanism of the cells to fight the intruders [95, 102]. On the other hand, *C. pneumoniae* is able to survive in that oxidative environment, which might induce their persistent form and thus facilitate their prolonged survival in the host cells [97].

ROS production in THP-1 cells was measured in this work, and both LPS and *C. pneumoniae* infection induced the intracellular ROS levels in these cells after 48 h exposure (Figure 7). The impact of schisandrin, schisandrin B and schisandrin C was detected on basal as well as LPS- and *C. pneumoniae*-induced ROS levels. Schisandrin elevated the basal ROS levels already at a 1-h exposure in THP-1 monocytes (Figure 8A), whereas schisandrin and schisandrin C did so after a longer, 24–72-h exposure in THP-1 macrophages (Figure 8B). Interestingly, the concomitant infection/LPS exposure reversed this lignan-induced elevation in naïve cells, and schisandrin B even decreased the ROS levels in LPS- (Figure 9A) and *C. pneumoniae*-stimulated samples (Figure 9B). Schisandrin C caused a similar decrease in ROS only in LPS-induces samples (Figure 9A). Of note, schisandrin B decreased the THP-1 macrophage cell viability significantly (Table 8), which may have an impact on these results.

The ROS levels, as detected by the DCFH-DA method, represent the total ROS amount in the cells. It remains unknown whether these ROS are produced in the mitochondrial respiratory chain or in the cytoplasm e.g. via NADPH oxidase, and further studies should be carried out in the future to elucidate the mechanisms of the observed ROS alterations.

Nitric oxide (NO) is one of the molecules related to *C. pneumoniae*-host cell interaction [97]. NO functions as a signaling molecule, and it plays an important role in host defense against bacterial infections [252]. NO itself is not particularly reactive, but more reactive nitrogen molecules, namely peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2) and nitrogen trioxide (N_2O_3) are produced upon its reaction with oxygen or superoxide. NO is produced by two different enzymes, endothelial nitric oxide synthetase (eNOS), which is constitutively producing NO maintaining the normal functions in the cells, such as vasodilation and proliferation, and inducible NOS (iNOS), which is expressed e.g. in macrophages in response to inflammation and cytokine release [253]. *C. pneumoniae* has been reported to induce NO production via iNOS, which leads to oxidative stress and thus inflammation in the cells [101, 254]. The results in this work demonstrate that both LPS (Figure 6A) and *C. pneumoniae* (Figure 6B) exposure elevate the levels of nitrate, the metabolite of NO, in RAW267.4 cells, but no elevation after several attempts

was observed in THP-1 cells. There have been some controversial results of this matter, but several studies support our findings that NO is not produced in THP-1 cells [233, 255]. In general, conflicting evidence of the production of NO in human PBMC has been presented among the studies [256]. These results imply that there are cell type- and species-specific differences in the production of NO after bacterial exposure, which further translates into differential inflammatory reactions. The impact of schisandrin and schisandrin B on LPS- (Figure 6A) and *C. pneumoniae*-induced (Figure 6B) NO levels were also studied in RAW264.7 macrophages. The results showed that schisandrin B, but not schisandrin, decreased both the LPS- and *C. pneumoniae*-induced NO levels significantly. These results suggest that schisandrin B alleviates the infection-induced inflammatory response in murine macrophages, at least by decreasing NO levels. The potential clinical relevance of this effect in *C. pneumoniae* infection in humans is uncertain due to the species-specific differences in NO production. NO, however, is produced by other human cells, such as epithelial and endothelial cells [257] and the effect of schisandrin lignans on *C. pneumoniae*-induced NO production in those cells should be studied further.

The effects of schisandrin lignans on the oxidative state in cells have been widely studied. There are several studies suggesting the increase of intracellular ROS/RNS by schisandrin lignans [219, 222, 258-260], while other studies report no such impact [203], or even opposing effects [261-263]. It seems that the nature of impact schisandrin lignans are exerting on the cellular redox state is strongly dependent on the cell type and the way the cells are stimulated/treated, and the mechanisms of action may also vary. In murine models, the effect of schisandrin B on the cellular redox balance has been suggested to occur via the inhibition of nuclear factor-erythroid 2-related factor 2 (Nrf2), which induces the production of antioxidative enzymes and further leads to inhibition of NF-κB function [219].

NF-κB is a transcription factor that regulates the gene expression involving inflammatory functions, such as that of the cytokines *IL-1, IL-6, IL-8* and *IL-12* [264]. The NF-κB activation is also considered as one of the hallmarks of chronic inflammatory

diseases [264]. It has been shown to have a major role in atherogenesis by regulating the transcription of inflammatory genes, cell proliferation, and apoptosis. NF-κB has been found to be activated in atheromatous, but not in healthy arteries [265]. *C. pneumoniae* infection also affects the NF-κB function via the TLR2/4-MAPK signaling pathway [139, 148]. The association of schisandrin lignans and *C. pneumoniae* with NF-κB could be one of the mechanisms leading to the alteration of ROS levels. However, confirming this hypothesis is beyond the scope of this work and will instead be the subject of a future project.

In this study, we examined whether THP-1 monocytes produced cytokines after LPS administration or *C. pneumoniae* infection. This cell line was found to secrete IL-6 and IL-12 after LPS exposure, but only IL-12 after *C. pneumoniae* infection, and the concentration was remarkably lower than that seen in the LPS-exposed cells. It is known that LPS of *C. pneumoniae* is not as immunogenic as LPS of other gram-negative bacteria [266], so these kinds of results were as expected and only confirmed the pre-made assumptions, about the different immunogenicity of *E. coli* and *C. pneumoniae.* There may be several factors explaining such low or absent cytokine levels in our experiments when compared to previously reported studies (Table 2). Firstly, the exposure to *C. pneumoniae* has been conducted differentially in the experiments. Mamata et al. [91], for example, used a MOI of 10 in their experiments, whereas we infected at a more moderate dose at MOI2. These authors also used a different strain of the bacterium, TW-183, in place of the K7 used in our studies, which could also result in a dramatically different immune response. Zhou et al. [93] studied the impact of isolated chlamydial Hsp10 protein on host cells, whereas all our experiments were conducted using whole bacterial infections. The more complete host-pathogen interplay captured in our data may also translate into contradictory results. Schisandrin lignans influenced inflammatory cytokine production (Figure 5) in both LPS- and *C. pneumoniae*-induced cells. We showed that schisandrin B and schisandrin C reduce the IL-6 and IL-12 production in THP-1 monocytes. This reduction may occur via the inhibition of the NF-κB function through the transcription factor Nrf2, but further

experiments must be conducted to verify this hypothesis. PPARγ is also known to suppress the NF-κB [130], and schisandrin B and schisandrin C might have inhibited the NF-κB function by inducing the *PPARγ* transcription (Table 10). Our gene expression analysis was carried out in murine cells, which should be taken into account when comparing the results gained from human cells. There are studies reporting the impact of schisandrin lignans on the production of inflammatory cytokines, namely IL-1β, IL-4, IL-6 and TNF-α, but most of these have been conducted in rodent models [219, 220] and only a few in human cell lines [267]. Our results provide novel information about the effects of schisandrin lignans in human cells and support the hypothesis that these lignans can alleviate the inflammatory response in human immune cells.

NF-κB has been reported to be involved in inflammasome activation [268]. Inflammasomes are protein complexes that activate caspases, which further activate inflammatory cytokine production. Inflammasomes are part of the innate immune system and play a significant role in defense against pathogens [269]. Nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) is the most widely studied inflammasome and its mechanism of action is well characterized. The activation of inflammasomes is a two-step process. First, in NLRP3 priming, the transcription of the inflammasome components caspase-1 and pro-IL-18 is upregulated, which occurs through different stimuli of cellular stress, e.g. by PAMPs that activate TLRs, further leading to NF-κB activation. ROS are also reported to be an important factor in activating the NLPR3 inflammasome [270]. Mitochondrial dysfunction and, consequently, mitochondrial ROS (mtROS) production are also playing a role in NLRP3 activation [271]. In addition to ROS, the regulator of antioxidant gene expression Nrf2 is known to take part in the activation of the NLRP3 inflammasome. On one hand, *Nrf2* expression can downregulate the NLRP3 activation by reducing NF-κB function, and through that the expression of *NLRP3*, *Caspase-1* and *IL-18* (the components of NLRP3 inflammasome) is also downregulated [272]. On the other hand, *Nrf2* expression is reported to be necessary for inflammasome activation [273].

C. pneumoniae infection has also been reported to activate the NLRP3 inflammasome [274]. It has been shown that *C. pneumoniae* increases IL-1β production, which requires the NLRP3 inflammasome activation in murine macrophages [275, 276]. In addition, *C. pneumoniae* growth has been shown to be reduced without NLPR3 inflammasome activation [274], implying that it is beneficial for the bacteria. Studies have also showed that lipid accumulation is related to NLRP3 inflammasome activation and the following inhibition of ABCA1-mediated cholesterol efflux [276].

Schisandrin lignans are known to activate Nrf2 and, through that, the NF- κB function is reduced [219]. Nrf2 has also been reported to inhibit NLRP3 activation and IL-1β production [272]. Indeed, schisandrin B has been demonstrated to suppress the NLRP3 inflammasome through *Nrf2* expression [277]. Thus, one mechanism by which schisandrin B and schisandrin C alleviate the *C. pneumoniae*-induced inflammatory effects in host cells could be by inhibiting NLRP3 inflammasome activation through Nrf2 and NF-κB. This may in turn lead to ameliorated inflammatory reaction, which could also result in the inhibition of lipid accumulation and thus the reduction of foam cell formation also observed in this study.

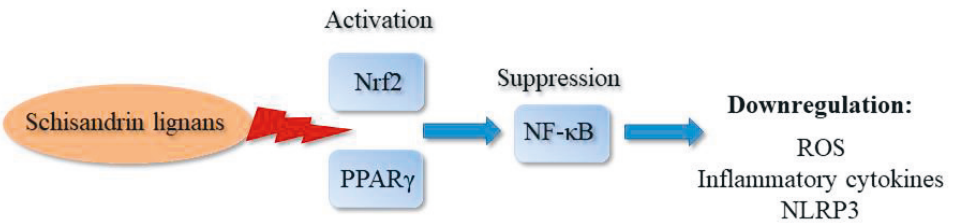


Figure 15. The possible signaling pathway involved in the activity of schisandrin lignans in macrophages, leading to alleviation of inflammation and atheroma formation. Schisandrin lignans activate Nrf2 and PPARγ, which suppresses the NF-κB signaling and that leads to the downregulation of the inflammatory cytokine production, NLRP3 activation and ROS production. This alleviates the oxidative stress and consequently also the foam cell formation could be inhibited.

In addition to NF-κB, another transcription factor that is regulated by *C. pneumoniae* infection is PPARγ. PPARγ is a nuclear receptor, which regulates the lipid and glucose metabolism as well as immune responses in the cells [127]. *C. pneumoniae* affects PPARγ activation via the MAPK pathway [149] which leads to alterations in cellular lipid metabolism. PPARγ is also associated with glutathione metabolism in the cells [278, 279], which is required for the regulation of cellular oxidative state. In turn, this is reflected to lipid metabolism and the overall inflammation status.

The glutathione (GSH) system is one of the main regulators of oxidative homeostasis in the cells. GSH is synthetized by two reactions catalyzed by glutamate-cysteine ligase (GCL) and GSH synthetase (GS) [103]. First, GCL forms γ-glutamylcysteine from glutamate and cysteine, which is the rate limiting step in GSH synthesis and depends on the availability of cysteine. GCL contains two subunits, a catalytic subunit, GCLc, and a modifier subunit, GCLm. In addition to the depletion of cysteine, the synthesis rate is also inhibited by a feedback mechanism by GSH. The other reaction in GSH synthesis is the condensation of γ-glutamylcysteine and glycine, which is catalyzed by GS.

Various cellular factors regulate GSH synthesis via GCL activity, e.g. oxidative stress, NF-κB activity, inflammatory cytokines and NO production [103]. One important regulator of the GSH synthesis rate is the activation of γ-glytamyltranspeptidase (GGT-1), which is the only enzyme that can break down GSH [280]. GGT-1 is located on the outer cell membrane where it breaks down GSH to γ-glutamyl compounds and cysteinylglycine, which is further cleaved into amino acids. These amino acids, including cysteine, are then taken back into the cells and used in GSH synthesis, thus making GGT-1 a critical component of the cellular GSH homeostasis.

Oxidative stress is expected to decrease the levels of GSH because it is used to scavenge the oxidants from the cells. The decrease in overall GSH leads to the upregulation of its synthesis: GGT-1 levels become elevated, which increases the extracellular GSH breakdown and intracellular GSH synthesis, leading to the protection of the cells against oxidative stress.

On a systemic level, GSH can be transported between the tissues where it is needed to counteract oxidative stress or exert other effects. The GSH is then exported out from the cells, broken down by GGT-1, and transferred wherever the components are needed for GSH synthesis [281].

In this work, the impact of *C. pneumoniae* infection on GSH levels in THP-1 macrophages was studied. The infection increased the cellular GSH levels at 48 and 72 h of incubation. In previous reports, *C. pneumoniae* has been shown to reduce the GSH levels in RAW264.7 [99] and human T cells [114], but no data were available from the THP-1 cell line. The results from this work, together with previous findings, suggest that the impact of *C. pneumoniae* infection on cellular GSH levels are species- and cell type specific.

In addition, the impact of schisandrin, schisandrin B and schisandrin C on basal intracellular GSH was studied in both monocytic and macrophage-like THP-1 cells as well as in RAW264.7. Interestingly, the results were markedly different between the human and murine cell lines (Figure 10). In human THP-1 monocytes and macrophages, schisandrin B and schisandrin C decreased the total GSH levels at all time points, whereas in RAW264.7 macrophages schisandrin B and schisandrin C elevated the total GSH levels, and schisandrin decreased the levels after 72 h incubation. These results further support the finding that there are species-specific differences in the GSH signaling cascade between mice and humans. Furthermore, they demonstrate that schisandrin lignans cause different, even opposite results between different cell lines, which is an important factor to consider in the interpretation of other studies on these compounds. The sensitivity of cells towards these studied schisandrin lignans also varies between the cell lines (Table 8). Schisandrin and schisandrin C are well tolerated by cells used in these studies, but schisandrin B decreases the viability of THP-1 macrophages and RAW264.7 macrophages with high concentration and long incubation time.

We also examined whether schisandrin lignans affected the expression of genes involved in the GSH metabolism in RAW264.7 cells. The transcription levels of *GCLc*, *GCLm,*

and *GGT-1* were determined. Schisandrin B was shown to cause the upregulation of each of these genes (Table 10), which implies that GSH synthesis was increased in schisandrin B-treated cells, a finding that is in line with the observed elevation of the total GSH levels (Figure 10). Interestingly, only the transcription of *GCLm* was changed in schisandrin C-treated cells. This could mean that the observed protein levels arise from post-transcriptional control. Moreover, the finding implies that schisandrin B and C may have different mechanisms for the alteration of cellular GSH levels. The gene expression profile in THP-1 cells would be an interesting subject for a future study, since the impact of schisandrin lignans on GSH levels in THP-1 cells were opposite to the ones seen in RAW264.7 macrophages.

Oxidative stress is a major pathological mechanism in the development of atherosclerosis [282]. Atheroma formation begins when macrophages accumulate lipids within their cytoplasm and thus become foam cells. The foam cells then gather in the arterial walls and attract other cells and components, which further enhances the atheroma formation [283]. Lipid oxidization is a crucial step in foam cell formation [282] and intracellular ROS are involved in the signaling pathways induced by the recognition and internalization of oxidized LDL [284].

We studied the foam cell formation in THP-1 and RAW264.7 macrophages, and *C. pneumoniae* in the presence of LDL was shown to clearly promote lipid accumulation in both cells lines (Figure 12). There are several studies reporting the foam cell formation after *C. pneumoniae* infection in human macrophages [134, 137, 138, 140], mouse macrophages [131-133] and also in *in vivo* animal models [141, 276]. In this study, gene expression analyses were conducted to investigate the possible mechanisms related to *C. pneumoniae*-induced lipid accumulation in RAW264.7 cells. When the transcription of genes related to lipid metabolism (*PPARγ*, a transcription factor; *CD36*, scavenger receptor; *ABCA1*, cholesterol efflux protein) during *C. pneumoniae* infection was quantified, the transcription of *PPARγ* was observed to be significantly reduced (Table 9). A similar *C. pneumoniae*-induced *PPARγ* reduction has previously been reported in THP-1 cells [140] but no data have been available in RAW264.7 macrophages.

According to these data, it seems that *C. pneumoniae*-stimulated foam cell formation in RAW264.7 macrophages is associated with *PPARγ* downregulation.

The impact of schisandrin lignans on RAW264.7 and THP-1 foam cell formation and their ability to alter the gene expression in RAW264.7 cells, concerning lipid metabolism, were also studied. Schisandrin had no effect on lipid accumulation in either cell line (Figure 12), but schisandrin B and schisandrin C reduced it, thus inhibiting foam cell formation in both. It is of note, however, that the lipid accumulation was only observed by microscopy and not quantified by other methods. Both schisandrin B and C also showed a tendency to suppress the degree of cholesterol esterification in RAW264.7 macrophages (Fig 13).

The gene expression analyses demonstrated that schisandrin B and schisandrin C induced the *PPARγ* transcription in RAW264.7 cells (Table 10), which can be one mechanism behind the alleviation of lipid accumulation as shown in Figure 12. Schisandrin, instead, had no effect on the *PPARγ* transcription and it also did not inhibit the foam cell formation, providing further evidence for this association. We also examined whether foam cell formation inhibition by schisandrin B and C could occur due to the expression of genes involved in lipid uptake (*CD36*) or lipid efflux (*ABCA1*) (Table 10). There were no statistically significant changes in *CD36* transcription after treatment with the lignans. Even though CD36 has been reported to be important in oxidized LDL uptake, its role in atherogenesis is controversial [285]. Another scavenger receptor, lectin-like oxLDL receptor-1 (LOX-1), is also known to take part in oxidized LDL uptake [282] and further studies on its role in the impact of schisandrin lignans on foam cell formation might shed more light on the question. On the other hand, the CD36 regulation may also occur on the translational level, which could explain its unaltered gene expression. Indeed, although it has been reported that *CD36* transcription is induced via a PPARγ-dependent manner [286], other studies suggest that PPARγ activation is not needed for *CD36* upregulation [287].

The transcription of the cholesterol efflux pump gene, *ABCA1* was significantly affected by treatment with the schisandrin lignans. Schisandrin downregulated *ACBA1* transcription (Table 10), indicating that lipid efflux was decreased in the cells. This may result in increased lipid accumulation, but the matter requires further investigation. Schisandrin B and schisandrin C, instead, upregulated *ABCA1*, indicating that lipid efflux may have been increased in the cells, which could explain the observed reduction in foam cell formation. In the foam cell studies, the impact of MAPK inhibitors on the lipid accumulation of RAW264.7 and THP-1 cells was also determined. The JNK inhibitor SP600125 prevented the lipid accumulation in RAW264.7 cells (Figure 12A) but not in THP-1 cells (Figure 12B), whereas the p38 inhibitor SB203580 seemed to reduce the lipid accumulation in THP-1 cells. These results are in line with previous observations that the foam cell formation occurs via the MAPK pathway [132, 147, 149], and further imply that there are cell-type or species-specific differences in the kinases involved in the process. The impact of GSH synthesis inhibitor BSO on foam cell formation in RAW264.7 cells was also studied, and it was shown to induce lipid accumulation in these cells (Figure 12A). This implies that the GSH concentration in the cells is strongly associated with foam cell formation in RAW264.7, which has also been suggested in previous studies [234]. One possible mechanism for the schisandrin B and schisandrin C-mediated elevation of the GSH concentration could be the induction of GSH synthesis and the resulting alleviation of the oxidative stress in the cells. In THP-1 cells, the mechanisms concerning the alteration of GSH levels and foam cell formation seems to differ from that in RAW264.7 macrophages, since the schisandrin lignans reduced the cellular GSH levels but still seemed to inhibit foam cell formation.

Monocytes and macrophages are key mediators of chlamydial infection and its contribution to various chronic inflammatory diseases. One of the most efficient ways to decrease *C. pneumoniae*-related infection burden in chronic inflammatory diseases would be to block the transfer of bacteria from lung epithelium to phagocytes, which would eliminate the possibility of the bacteria to initiate the inflammatory processes leading to diseases such as atherosclerosis. In one of the studies included in this thesis,

a novel platform was established for studying the transfer of *C. pneumoniae* from epithelial HL cells to THP-1 monocytes. The method is based on the efficient separation of THP-1 cells from previously infected HL cells after their coculture, followed by the quantification of *C. pneumoniae* genome numbers in the THP-1. Despite the well-described role of systemic *C. pneumoniae* dissemination in chronic inflammatory diseases, there is a very limited amount of studies demonstrating the use of coculture models in *Chlamydia* research. U937 monocytes have been cultured together with infected human arterial endothelial cells (HMECs) [288], human umbilical vein endothelial cells (HUVECs) and coronary artery smooth muscle cells (CASMCs) [289]. These studies reported that *C. pneumoniae* is able to transfer from monocytes to HMECs and CASMCs. However, these studies concern a later stage of systemic *C. pneumoniae* dissemination, in which bacteria have already transferred from the respiratory tract to monocytes and were then further transferring to the vasculature. Before this work, no studies of *C. pneumoniae* transfer from epithelial cells to phagocytes were available.

C. pneumoniae has been reported to activate the MAPK signaling pathway in the cells [248, 290], and this pathway is important e.g. in the internalization and exit of the bacteria [40, 291]. *C. pneumoniae* is thought to activate the MAPK pathway through TLR binding. When TLRs recognize *C. pneumoniae*, the JNK, ERK and p38 kinases are phosphorylated and activated immediately [248]. MAPK signaling activates actin polymerization and remodeling at the site of *C. pneumoniae* entry, which enables the successful internalization of the bacteria [291]. This is also supported by the finding that the blockage of MAPK signaling decreases *C. pneumoniae* internalization in epithelial cells [40]. Besides the TLR-mediated MAPK activation, another factor related to actin remodeling and *C. pneumoniae* internalization is the chlamydial protein Tarp [56]. It has been reported to be involved in MAPK signaling activation [292], and Tarp might thus be one means of *C. pneumoniae* to induce actin remodeling.

While optimizing the coculture model, the MAPK inhibitors, SP600125 (JNK), SB203580 (p38) and FR180204 (ERK) were studied as possible positive controls to be used in the assay. All three MAPK inhibitors inhibited the *C. pneumoniae* transfer

between the epithelial cells and monocytes (Figure 13). In several follow-up studies, we investigated at which point of the transfer the MAPK inhibitors might be interfering. Our results show that the JNK inhibitor (SP600125) and p38 inhibitor (SB203580) were able to significantly inhibit the *C. pneumoniae* internalization (Figure 14). The ERK inhibitor also reduced internalization, but the change did not reach statistical significance. Actin polymerization is also necessary in one of the exiting mechanisms of *Chlamydia*, extrusion. When newly-formed EBs inside the host cells are ready to exit, actin is polymerized and recruited to form a coat around the bacteria, which enables the inclusion to bud out from the cells leaving the host cell intact [293]. The impact of MAPK inhibitors on *C. pneumoniae* exit could be one possible mechanism by which they block the transfer. Although there was no reduction in EB release from the HL epithelial cells with any of the studied MAPK inhibitors (Figure 14), the JNK inhibitor was shown to induce the EB exit. This is an interesting result and should be examined in further studies. These results also demonstrate that there were no changes in either the EB production in HL cells or the EB infectivity after MAPK treatment. Taken together, these results show that MAPK inhibitors can be used as positive controls in the studies of *C. pneumoniae* transfer using the described novel method. The overall impact of MAPK inhibitors on *C. pneumoniae* transfer remains unknown, with one identified mechanism being their ability to block the internalization of bacteria into THP-1 cells. This novel platform provides new opportunities for further profiling studies of schisandrin lignans as well as other antichlamydial compounds in the future.

7. Conclusions

In this work, monocyte and macrophage cell lines were used in *C. pneumoniae* infections to establish cell models harboring persistent chlamydial infection. The impact of antichlamydial dibenzocyclooctadiene lignans schisandrin, schisandrin B and schisandrin C was evaluated on the *C. pneumoniae*-induced inflammatory functions in these cell lines. These studies showed that *C. pneumoniae* modifies the normal functions of these innate immune system cells. A varying activity of the studied lignans on these inflammatory functions was additionally demonstrated.

While *C. pneumoniae* infection initiated the inflammatory functions of the cells, including inflammatory cytokine production, redox balance alteration and foam cell formation, schisandrin B and schisandrin C showed an alleviating action against all of those cellular functions. Of the three studied lignans, schisandrin was least effective against *C. pneumoniae*-induced inflammatory functions.

Differential responses between human THP-1 and murine RAW264.7 cell lines were observed concerning both the *C. pneumoniae* infection and the schisandrin lignan treatment, implying species-specific differences in the innate immune system. There was no NO production recorded in THP-1 cells in our studies, but the NO levels in RAW264.7 were significantly elevated during *C. pneumoniae* infection. The GSH signaling pathways also seem to act differently between the cell lines, since the schisandrin lignan treatment resulted in opposing responses. In addition, the MAPK signaling pathways seem to activate differently between the cell lines. In the foam cell studies, the JNK inhibitor only blocked the lipid accumulation in RAW264.7 cells and not in THP-1 cells, while the p38 inhibitor showed activity in THP-1 cells. Considering these differences, it may be advisable to prioritize the use of human cell lines in the *C. pneumoniae* studies aimed for the development of new antichlamydial lead compounds.

The overall aim behind the studies in this work, conducted with schisandrin lignans in innate immune system cells, was the profiling of the compounds against persistent *C.*

pneumoniae infection. In addition to the eradication of *C. pneumoniae* from the tissues and diminishing its inflammatory functions, blocking the transfer of the bacteria from respiratory tract epithelium to phagocytes could be a putative mechanism to fight the chlamydial persistence. In this thesis, I introduced a novel platform for studying the *C. pneumoniae* transfer between the cells, which could be added to the compound profiling workflow of antichlamydial compounds.

Both *in vitro* and *in vivo* studies of absorption, distribution, metabolism and excretion should be conducted with schisandrin lignans, as well as *in vivo* toxicity studies. If the results from those will be satisfactory, clinical trials may be planned and the drug development of these natural compounds continued.

The results presented in this thesis have provided a lot of new information about schisandrin lignans and their mechanisms against *C. pneumoniae*-induced inflammatory functions and atheroma progression. Altogether, these studies have highlighted the potential of schisandrin lignans as antichlamydial compounds and increased the knowledge on the related pathological processes.

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